University of Alberta

Caspase-Independent CTL-Mediated Killing

by

Jeffrey Alexander Heibein

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A thesis submitted to the Faculty of Graduate Studies and Research in Partial Fulfillment of the requirements for the degree of Doctor of Philosophy

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Evolution is cleverer than you are.

-Leslie Orgel

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read and recommended for acceptance to the Faculty of Graduate Studies and Research a thesis entitled **'Caspase-Independent CTL-Mediated Killing'**. Jeffrey A. Heibein has submitted this thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Cytotoxic T lymphocytes (CTLs) are responsible for eliminating virus-infected cells and tumour cells and contribute to the rejection of transplanted organs. CTL eliminate targets by inducing them to die through apoptosis, a physiological process of cell death in which the cell actively participates in its own demise. Apoptosis is initiated following activation of members of the caspase family of cysteinyl proteinases. Other features characteristic of apoptosis are the disruption of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) and the release of apoptogenic proteins, including cytochrome c, from mitochondria into the cytoplasm. Mitochondrial events appear to occur early in many systems in response to a variety of apoptotic stimuli and lead to the activation of caspase-3 and subsequently, apoptotic morphologies.

CTLs have two mechanisms at their disposal for eliminating targets. The first involves binding of the Fas receptor on the target cell surface by Fas Ligand expressed on the surface of the CTL. The second mechanism involves granule exocytosis. Exocytosed granule contents, including granzyme B and perforin are taken up by the target and subsequently induce apoptosis. Granzyme B-dependent apoptosis was believed to result exclusively from direct processing of caspase-3 by granzyme B. However, reports have surfaced suggesting that additional caspase-independent mechansims of granzyme B-mediated killing exist.

This thesis provides evidence that both $\Delta\Psi_m$ collapse and cytochrome c release occurs through a caspase-independent mechanism in targets in response to granzyme B-mediated killing. Cytochrome c release requires the direct proteolytic cleavage of the Bcl-2 family member Bid by granzyme B to generate a 14 kDa granzyme B-truncated product (gtBid) which translocates to mitochondria. In turn, gtBid recruits Bax to

mitochondria through a process antagonized by the anti-apoptotic Bcl-2. Bax integrates into the mitochondrial membrane and induces cytochrome c release through an unknown caspase-independent mechanism. Data presented in this thesis also indicate that while granzyme B can directly process pro-caspase-3, acquisition by caspase-3 of full enzymatic activity following this processing requires mitochondrial events that are antagonized by Bcl-2. Our results provide evidence for a new pathway by which CTL inflict target cell damage and additionally provide an explanation for granzyme B-dependent, caspase-independent CTL-mediated killing.

Acknowledgements

I doubt very much that many students recognize the magnitude of the journey upon which they embark when deciding to begin graduate studies. I certainly didn't. Although I had done research prior to applying for graduate school and felt comfortable with the notion of being a scientist, in retrospect I now see that I was extremely naïve about how science worked. I don't simply mean that I didn't understand how to do research, though over the last four years I have unquestionably learned a great deal about how research is done. Rather, I mean that science is a complicated entity. During my high school days and even throughout my undergraduate years, I had come to believe that science was a completely objective approach to understanding the world around us. Science was a process of discovery allowing concrete statements to be made concerning the unknown. One collected data, examined that data and based upon that data, the fact either was or wasn't.

I now see that science is more art form that mere objective cataloguing. It is true that as scientists we collect data and objectively attempt to understand this information. If all data yielded black and white answers to all our questions, this would be a relatively simple task and I rather suspect that nearly anyone could become a successful scientist. The reality, however, is that the answers to our questions are rarely black and white. By contrast, what we term "understanding" usually means interpreting data with respect to the larger pool of knowledge and in terms of our expectations and biases. When put into this context, interpretation is anything but objective as we inevitably cling to ideas that make sense to us. Interpretation requires a keen intellect as well as a flash of creative genius and successfully combining these elements is no easy assignment. The mere fact that students begin to hone their skills of scientific inquiry well before entering university attests to the long and at times arduous task of developing sound scientific approaches to understanding our world.

I can make no conclusive statement as to my current skills as a researcher. I leave that to my peers. Nevertheless, I have learned a great deal about how to do research while at the University of Alberta. The journey has been a collective venture and many individuals have contributed to shape my philosophy of science. However, Chris Bleackley has on balance, largely influenced my approach to science. I suppose this is not strange considering the duration of time I have spent toiling in his lab. But I truly respect Chris as a researcher and a mentor and I have tried to learn all I can before I move on. Chris not only taught me much about how to ask questions and how to interpret the answers, he has also taught me how to share that knowledge. For if as scientists we are unable to share our findings in a meaningful way, why seek them out at all? He has also allowed me to make my own choices about which experiments were the best or, as in some cases, the experiments that were clearly not the best, but he has let me make my own mistakes. That freedom was invaluable and has amply prepared me for things to come. For all this, I thank Chris.

I must thank in especial, Ing Swie Goping and Michele Barry. Both taught me a great deal about scientific interpretation and the organization of publications. Some of the data

appearing in this thesis was kindly provided by these two individuals (where noted) and for that I offer heart-felt thanks. Charolyn Babichuck, Tony Caputo, Marita Hobman, Bruce Motyka and Kirstin Veugelers have also offered many suggestions and comments that have helped direct my research. Collectively, the laughter and merriment of all these individuals has also made the lab just plain fun, and such an atmosphere cannot help but stimulate quality science.

Eric Atkinson was the first to teach me a [^{3}H] thymidine release assay. I admire Eric for his scientific brilliance and his grumpy wit but it was Eric's vast collection of personal scientific disasters that quickly put me at ease with my own mistakes during the initial obligatory failure period all grad students must endure when entering a new lab.

Michael Pinkoski was always good for a cup of coffee. Or rather, he was always searching for a cup of coffee. It has been my experience that many, if not most, scientific ideas of any quality evolve from group discussions. Given this philosophy, the endless quest for coffee and the discussion that invariably accompanies it once found is simply an extension of the scientific process. I must thank Pinky for bringing me to this realization and I shall always carry the quest for coffee to whatever problem solving the future requires.

The few molecular biology skills I now possess I attribute to Brenda Duggan and Nancy Ehrman. These two persons, denizens of the lab, have also brought much joy with their endless tales of days gone by when real women were real women, real men were loathsome, and there was no use crying over spilt mitochondria

Tracy Sawchuk, Irene Shostak and Diane Marganski deserve more thanks than can be heaped into such a page as this. In addition to their seemingly endless supply of mirth, they are essential cogs within the machinery of the lab. If any of them was to leave, I fear that operations would quickly grind to a halt. Over the years, they have provided cell culture support in particular but also they have provided a foundation upon which rests the smooth day to day running of the lab. This has allowed all of us to do our jobs more efficiently and more enjoyably. Saying 'thank you' to these three doesn't even cover it.

Although my peculiar brand of speaking style stems mostly from Monty Python albums listened to as a child while building castles out of Lego, it was Samantha James who helped me to find within myself the confidence to get the story out. She taught me that if confidence couldn't be truly exuded, it could at least be faked and for that I'll always be grateful.

Save for the acquisition of an earring at age 16, I cannot think of a time when both my parents did not offer their whole-hearted support for any endeavor I chose. As I child I naturally took this support for granted, as doubtless most of us do, and I'm certain that I have at the very least contributed to my parents collective accumulation of gray hairs. But without them (my parents, not the gray hairs), I'm convinced that I would not have had many of the opportunities that have come my way and I cannot imagine what type of person I would be today without their love and support. Without question, I would not practice science with the broad-minded approach that I currently favour. And so to my parents I must apologize for the undue stress my childhood surely caused them, and I must give thanks for 31 years of guidance, support and love.

I have experienced a peculiar sorrow in writing these acknowledgments. Another of life's chapters is drawing to a close. Since this chapter was an exceptionally good one, it saddens me that it will soon end. I shall take with me all that I have learned here and I can only hope that the next chapter is as entertaining.

Thank you all.

JAH, December 2000

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List of Abbreviations

AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocator
APC	antigen presenting cell
BIR	baculoviral IAP repeat
C	carboxy
CAD	caspase-activated DNase
caspase	cysteinyl aspartate-specific proteinase or cysteinyl aspartate-specific proteinase
CD	cluster of differentiation
CD95	cluster of differentiation 95; equivalent to Fas
CrmA	cytokine response modifier A
CsA	cyclosporine A
CTL	cytotoxic T lymphocyte or cytotoxic T cell
DASPEI	2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide
DCI	3,4-dichloroisocoumarin
DFF-45	DNA fragmentation factor-4
$DiOC_6(3)$	3,3'-dihexyloxacarbocyanine iodide
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

ECL	enzyme-linked chemiluminescence
EF	embryonic fibroblast
EM	electron microscopy
ER	endoplasmic reticulum
ES	embryonic stem cell
FADD	Fas-associated protein with a death domain
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HCTL	human CTL
HE	hydroethidine
HU	hemolytic units
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-activated DNase
ICE	interleukin-1β converting enzyme
Ig	immunoglobulin
IL	interleukin
JpEGFP	Jurkat stable transfectants containing the pEGFP-N1 plasmid
JpEGFPneo	Jurkat stable transfectants containing both the pEGFP-N1 and BMGneo (empty vector) plasmids
JpEGFPBcl-2	Jurkat stable transfectants containing both the pEGFP-N1 and BMGneo (containing a Bcl-2 cDNA insert) plasmids
mClCCP	carbonyl cyanide m-chlorophenyl hydrazone

MHC	major histocompatibility complex
MOI	multiplicity of infection
MOPS	morpholino propane sulphonic acid
MORT1	mediator of recptor-induced toxicity 1
N	amino
NK cell	natural killer cell
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PBST	PBS + 0.1% (v/v) Tween-20
pfu	plaque-forming units
PPIX	protoporphyrin IX
PT pore	permeability transition pore
RHFM	RPMI-1640 medium + 10% FBS + 100 μM 2-mercaptoethanol
RBL	rat basophilic leukemia cell
ROS	reactive oxygen species
SDS	sodium dodecylsulphate
T_c	T cytotoxic lymphocyte or T cytotoxic cell
TCR	T cell receptor
T_H	T helper lymphocyte or helper T cell
TNF	tumour necrosis factor

TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end-labeling
VDAC	voltage-dependent anion channel
zDEVD-fmk	benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone
$\Delta\Psi_m$	mitochondrial inner membrane potential

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Overview of the Immune System

The human immune system is comprised of two general arms. Innate (nonspecific) immunity includes physical and physiological barriers such as the skin and cellular mechanisms of pathogen removal such as phagocytosis by monocytes or macrophages. In addition, through the release of cytotoxic substances, granulocytic cells (neutrophils, basophils, eosinophils and mast cells) contribute immediate and nonspecific protection against pathogens that have breached physical barriers. The immunity offered by these cells is non-specific in the sense that it is not induced in response to, nor is it directed against specific antigens.

The second major arm of the immune system involves adaptive (specific) immunity. Unlike the immediate and non-specific nature of innate immunity, the adaptive response often requires many days to reach fruition. However, while it may be initially slow to evolve, an adaptive immune response is induced by the recognition of and response to specific antigen(s). Hence the power of an adaptive response is its ability to target and eliminate a specific array of antigens against a backdrop of non-offending signals. Cells of adaptive immunity are primarily lymphoid cells and include both T lymphocytes and antibody-producing B lymphocytes.

The specificity of the adaptive response is governed by interactions between antigen and specific receptors for that antigen found on the surfaces of lymphoid cells. B lymphocytes express the B cell receptor while T lymphocytes express the T cell receptor (TCR). During the maturation of lymphoid cells, the B or T cell receptors are generated through a complex series of gene rearrangements. This process yields a tremendous variety of receptor configurations, each recognizing antigen of a particular shape. Large numbers of these receptors are expressed on the surface of any given B

or T cell, but each B or T cell expresses receptors of only a single specificity. Thus each mature lymphoid cell is armed for recognition of a single antigen but the variety of lymphoid cells with different receptor configurations allows the immune system to respond to a seemingly limitless number of foreign antigens.

The adaptive immune system can be subdivided based upon the functions of the different lymphoid cells. B lymphocytes make up the humoral branch of adaptive immunity becoming activated following recognition of antigen by the B cell receptor. Activated B lymphocytes clonally proliferate to become antibody-secreting plasma cells and memory B cells. Antibodies, or immunoglobulins, are soluble versions of the B cell receptor that circulate in the blood or lymph to seek out foreign antigens. Antigen recognition and binding serves either to neutralize the harmful functions of the antigen or to signal to other immune cells such as macrophage that the antigen should be phagocytosed. Memory B cells, on the other hand, continue to express membrane-bound immunoglobulin receptors and remain as a reminder of the offending antigen to allow for a quicker response in the future should that antigen re-appear. In addition to antigen stimulation, efficient B cell proliferation also requires further stimulation by T cell-derived cytokines.

Unlike B lymphocytes which recognize antigen outright, T lymphocytes respond to antigens only when those antigens are presented in the proper context. T lymphocytes are divided into two categories based upon the manner in which antigen must be presented to them and the way in which they respond to presented antigen. The two classes of T lymphocytes are T helper (T_H) cells, which are required for initiating both humoral and cell-mediated responses, and T cytotoxic (T_C) cells, which are the effectors of cell-mediated immunity.

In the case of T_H cell stimulation, antigen presenting cells (APCs: B cells, macrophages or dendritic cells) must first engulf and digest exogenous (foreign)

protein antigen (**Figure 1.1-1**). Digested peptides are then packaged with class II major histocompatibility complex (MHC) and presented on the surface of the APC. The TCR of the T_H cell recognizes antigen presented with class II MHC but in addition, recognition requires the presence of a surface molecule called CD4 that acts as a co-receptor in conjunction with class II MHC. Although not absolutely exclusive, $CD4^+$ T_H cells are said to be class II restricted in that they only recognize antigen presented in the context of class II MHC. Binding by the TCR of an antigen presented in the context of class II MHC stimulates the $CD4^+$ T_H cell to clonally proliferate and secrete cytokines that activate and enhance both the humoral and cell-mediated responses. Additionally, some of the expanded T_H cell population differentiate to become memory T cells that function similarly to memory B cells, allowing the immune system to rapidly recognize and respond to a future appearance of a previously encountered antigen.

T_C cell activation works similarly to T_H cell stimulation, however T_C cells recognize antigen presented in the context of class I MHC (**Figure 1.1-1**). In addition, T_C cells express CD8 on their surface. Like CD4 on T_H cells, CD8 on the T_C cell is required for MHC I/peptide recognition and renders T_C cells class I MHC-restricted. Activated T_C cells are referred to as cytotoxic T lymphocytes or CTL. Unlike class II MHC that is expressed mainly on the surface of professional APCs, class I MHC is found on virtually all nucleated cells. In essence then, CTL monitor the health of all cells of the body. As with activated T_H cells, CTL activation leads to clonal expansion and cytokine production that fosters additional immune activation. Most importantly, however, CTL adopt the role of killer by seeking out and eliminating targets presenting altered self antigens in conjunction with class I MHC. Typically, such targets are virus-infected cells, tumour cells and the cells of foreign tissue grafts.

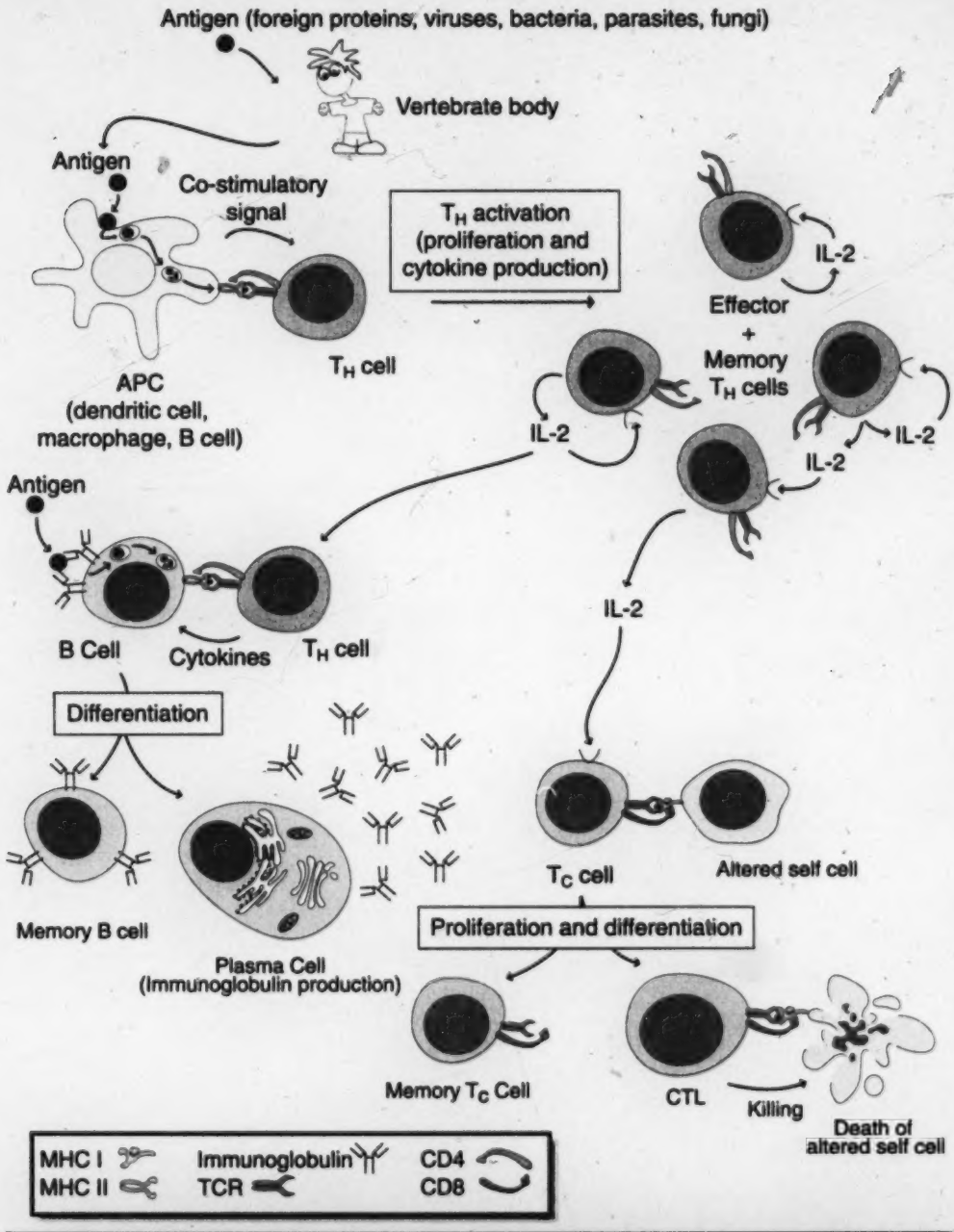

Figure 1.1-1 The Adaptive Immune Response

The immunological effects of antigen stimulation are shown. The major function of B cells is to produce antibodies while T_H cells stimulate the overall immune response. Stimulated T_C cells adopt a CTL morphology and effector function and are the functional effectors of cell-mediated immunity. Adapted from Kuby, Immunology, 3rd edtion.

Another class of lymphocyte responsible for cell-mediated immunity is the natural killer (NK) cell. NK cells function similarly to CTL in that they share the same killing machinery. However, NK cells do not have cell surface receptors allowing them to recognize specific antigen and so do not require antigen presentation. NK cells are therefore not MHC-restricted and are believed to kill targets through at least two mechanisms. First, although the exact nature of these interactions remains unclear, NK cells recognize antibody deposited onto the surface of tumours, which allows them to properly direct their killing machinery towards the tumour target. Second, NK cells can interact with tumour or virus-infected cells through direct membrane-membrane interactions to effect killing, although these mechanisms are poorly understood. NK cells are thought to be important for immunity against virus-infection or tumours during the lag periods when the B and T cell responses to antigen are in the early proliferative stages.

The sheer variety of human viruses and tumours which have evolved mechanisms for eluding cell-mediated surveillance underscores the importance of understanding the machinery employed by CTL and NK cells to effect target removal. Currently, the mechanisms underlying the killing process are poorly understood. This thesis focuses on one particular aspect of CTL-mediated killing and seeks to provide some understanding of how a CTL coerces a target cell into initiating its own demise. In purely intellectual terms, such work will add to our understanding of the immune system by adding another layer of complexity to an already bewilderingly complex system. More practically, however, a number of immune disorders including a variety of chronic virus infections and cancers involve an inability of CTL to eliminate certain types of pathogens and tumours. Hence, knowledge of the killing process will lead to more intuitive therapeutic interventions. Additionally, such understanding may permit

the design of improved immunosupressive therapies relative to those that are currently available.

1.2 Activation of a Killer: The CTL Response

In order for a CTL to kill a target, a complex series of events must occur in a particular sequence. A T_C cell must become activated. It must adopt a CTL effector morphology and the killing machinery within the CTL must be assembled and proffered to the target to ensure its efficient removal once it has been found. The next section outlines the events leading from T_C cell to CTL.

1.2.1 T_C Activation and the Induction of the CTL Response

Naïve or resting T cells were once thought to exist in a state of quiescence but recent studies utilizing gene array technology to examine mRNA expression under varying conditions suggest that these cells actively monitor and evaluate their environments (Teague et al., 1999). Proliferation of these cells is prevented by the expression of a number of genes that are thought to block progression into the cell cycle, including the retinoid acid receptor (RAR) and the proliferation inhibitory transcription factors D52 and TSC-22. On the other hand, the expression of interleukin (IL)-4, and -7 receptors, which play roles in proliferation and differentiation of T cells, remains high relative to their expression in activated T cells. Thus naïve T cells exist in an actively non-proliferative state but are primed for proliferation should the appropriate stimulation arise.

As mentioned in **section 1.1**, $CD8^+$ T_C cells adopt effector CTL function following the recognition of an appropriate antigen presented in conjunction with class I MHC. Two additional requirements must be met before full CTL function arises. The first is a co-stimulatory signal mediated through CD28 on the T_C cell interacting with the B7 molecule on an antigen presenting cell (June et al., 1994; Linsley and Ledbetter,

1993). The second is a signal induced by T_H-secreted IL-2 binding to the high affinity IL-2 receptor on the surface of the T_C cell (**Figure 1.1-1**).

Collectively, the signals outlined above stimulate signal transduction pathways causing changes in intracellular Ca^{2+} and phosphorylation levels (Esser et al., 1998; Esser et al., 1996; Rosato et al., 1994) leading to the transcription of a variety of genes, the expression of which culminates in effector CTL morphology and function (for review see Alberola-Ila et al., 1997; Cantrell, 1996). Included among these genes are a number of cell cycle proteins and the IL-2 receptor which foster proliferation (Teague et al., 1999). In addition, mRNA and protein levels are also upregulated for a number of secreted products of CTL that play roles in mediating the death signal such as granzymes, perforin (discussed in **section 1.3**) and Fas ligand (FasL) (discussed in **section 1.7.1**) (Atkinson and Bleackley, 1995; Teague et al., 1999).

Once activated, CTL are primed to kill. As with the stimulus required to cause activation of the T_C cell, TCR-dependent recognition of targets by activated CTL again demands presentation of the appropriate antigen complexed with class I MHC on the surface of a target cell (**Figure 1.1-1**). Though the specificity of binding between activated CTL and target is determined in large part by the TCR, class I MHC and co-stimulatory molecule interactions, it appears that additional molecules may be also important. Integrins expressed on the surface of the CTL interact with cadherins on the surface of the target cell to promote adherence between CTL and specific tissue types, virus-infected cells, or tumours (Hadley et al., 1997; Hadley et al., 1999; Schmits et al., 1996). Further, integrin binding stimulates phosphorylation events within the CTL that may be critical for degranulation (Ma et al., 1997) (degranulation is described in more detail in **section 1.2.2**). Once recognition has occurred, it is now generally accepted that elimination of the target transpires via one of two mechanisms (for review see Atkinson and Bleackley, 1995; Berke, 1994; Berke, 1995; Darmon et al., 1999; Shresta

et al., 1998; Trapani et al., 2000). The first involves binding of the Fas (CD95/APO-1) receptor on the surface of the target cell by Fas ligand expressed on the surface of the CTL (Fas-mediated killing is discussed in more detail in **section 1.7.1**). Binding of the Fas receptor and its subsequent oligomerization leads to the activation of signals within the cell instructing it to commit cellular suicide, a process known as apoptosis (a physiological process that will be examined in **section 1.4.1**). CTL also possess a second method for inducing apoptosis in target cells that involves the exocytosis of cytolytic proteins contained within CTL-derived granules. Through this mechanism, the target cell takes up a number of granule proteins that are believed to induce cell death by impinging upon pre-existing signaling pathways within the target (granule exocytosis is discussed further in the next section and **section 1.7.2**).

1.2.2 Cytolytic Granules

Many of the gene products upregulated during activation are stored in lytic granules within the activated CTL (Atkinson and Bleackley, 1995; Darmon et al., 1999). Morphologically, electron microscopy has shown the granules to resemble a dense core that is either surrounded by numerous small vesicles or by a small electron translucent halo (Vollenweider and Groscurth, 1991). The lytic granule has been described as a secretory lysosome since it shares features of both lysosomes and regulated secretory granules (for review see Griffiths, 1995). Ordinarily, the *trans*-Golgi separates proteins targeted to secretory granules from those destined for lysosomes and the two classes of proteins are packaged into distinct organelles (Burgoyne and Morgan, 1993; Kelly, 1985). However, in CTL, proteins of both secretory and lysosomal origins are sorted to the same organelle (Burkhardt et al., 1989; Burkhardt et al., 1990; Peters et al., 1991). A number of the known proteins found in cytolytic granules are listed in **Table 1.2-1**, illustrating the overlap of normally separately compartmentalized molecules. Proteins important for CTL-effector function such as the granzymes and

Table 1.2-1 Summary of Granule Contents

Protein	Known Function	Reference
CD63	Lysosomal protein	(Peters et al., 1991)
Arylsulphatase	Lysosomal enzyme	(Tschopp and Nabholz, 1990)
β-glucoronidase	Lysosomal enzyme	(Tschopp and Nabholz, 1990)
β-hexosamidase	Lysosomal enzyme	(Tschopp and Nabholz, 1990)
Calreticulin	Binds perforin; Ca^{2+} binding; chaperone	(Andrin et al., 1998; Burns et al., 1992; Dupuis et al., 1993)
Cathepsin D	Lysosomal protein	(Tschopp and Nabholz, 1990)
Chondroitin sulphate proteoglycans	Complex with perforin and the granzymes to inhibit function	[Tschopp, 1987 #264; Peters, 1991 #249; (MacDermott et al., 1985; Schmidt et al., 1985; Stevens et al., 1987)
Dipeptidyl peptidase I (cathepsin C)	Granzyme activation	(Caputo et al., 1993; Kummer et al., 1996; McGuire et al., 1990; Smyth et al., 1995)
Granulysin	Anti-microbial peptide	(Krensky, 2000; Pena and Krensky, 1997)
Granzymes	Proteolysis	See **Table 1.2-2**
H^+-ATPase	Granule acidification	(Kataoka et al., 1994; Kataoka et al., 1997)
Lamp-1	Lysosomal protein	(Peters et al., 1991)
Lamp-2	Lysosomal protein	(Peters et al., 1991)
Leukalexin	TNF-like cytokine	(Liu et al., 1987)
Leukophysin	Granule mobility	(Abdelhaleem et al., 1996; Abdelhaleem et al., 1991)
Mannose-6-phosphate receptor	Protein targeting	(Burkhardt et al., 1990; Griffiths and Isaaz, 1993; Peters et al., 1991)
Perforin (cytolysin)	Ca^{2+}-dependent pore-forming protein	(Groscurth et al., 1987; Podack et al., 1985; Young et al., 1986)
TIA-1	RNA binding	(Anderson et al., 1990; Tian et al., 1991)

Table 1.2-2 Summary of Known CTL-Specific Granzymes[1]

Granzyme	Synonym	Species	Enzyme Activity	Specificity (residue at S$_1$)	MW (kDa)	References
A	Hannuka Factor, SE1, CTLA-3, TSP-1, CTL Q31 granzyme 1, fragmentin-1	M, R, H	Tryptase	Arg/Lys	60[b]	(Brunet et al., 1986; Gershenfeld and Weissman, 1986; Hameed et al., 1988; Masson and Tschopp, 1985; Masson et al., 1986; Pasternack and Eisen, 1985; Poe et al., 1988; Shi et al., 1992; Simon et al., 1991; Young et al., 1986)
B	CCP1, SE2, CTLA-1, CGL-1, CSP-B, HLP, SECT, granzyme 2, fragmentin-2	M, R, H	Aspase	Asp/Glu	29-33[c]	(Brunet et al., 1986; Lobe et al., 1986; Lobe et al., 1986; Lobe et al., 1988; Masson and Tschopp, 1987; Shi et al., 1992; Young et al., 1986)
C	CCP2	M,R	?	Asn/Ser	27	(Jenne et al., 1988; Lobe et al., 1986; Lobe et al., 1986; Lobe et al., 1988)
D	CCP5	M	?	Phe/Leu	35-50[d]	(Bleackley et al., 1988; Bleackley et al., 1988; Jenne et al., 1988; Prendergast et al., 1991)
E	CCP3, MCSP-2	M	Chymase	Phe/Leu	35-45[d]	(Bleackley et al., 1988; Bleackley et al., 1988; Jenne et al., 1988; Kwon et al., 1988; Prendergast et al., 1991)

Continued on next page

Table 1.2-2 Summary of Known CTL-Specific Granzymes[f]

Granzyme	Synonym	Species[a]	Enzyme Activity	Specificity (residue at S_1)	MW (kDa)	References
F	CCP4, MCSP-3	M, R	Chymase	Phe/Leu	35-40[d]	(Bleackley et al., 1988; Bleackley et al., 1988; Jenne et al., 1988; Kwon et al., 1988; Prendergast et al., 1991)
G	CCP6	M	?	Phe/Leu	30	(Jenne et al., 1989; Masson and Tschopp, 1987)
H	CCPX	H	Chymase	?	30	(Edwards et al., 1999; Haddad et al., 1991; Meier et al., 1990)
K	granzyme 3, tryptase-2, fragmentin-3	M, R, H	Tryptase	Arg/Lys	30	(Hameed et al., 1988; Wilharm et al., 1999)

[f]The granzymes shown here are either expressed exclusively in CTL or are expressed in both CTL or NK cells. An additional granzyme, granzyme M (Metase-1), is expressed exclusively in NK cells. Abbreviations: M, mouse; R, rat; H, human. [a]Species in which genes have been cloned and/or proteinase isolated. [b]The active enzyme is a 60 kDa disulphide-linked homodimer (Bleackley et al., 1988). [c]Glycosylated. [d]Heavily glycosylated.

perforin are found alongside proteins traditionally considered lysosomal such as cathepsin D and arylsulphatase. The mannose-6-phosphate receptor, long established as a lysosomal marker, has been implicated in the proper trafficking of granzymes A and B to the lytic granule (Griffiths and Isaaz, 1993). However, other trafficking mechanisms must also exist for granule proteins as perforin is correctly localized to granules in CTL from patients suffering from I-cell disease (Griffiths, 1995; Griffiths and Isaaz, 1993). Persons with this disorder have a defect in the activity of a phosphotransferase consequently preventing the mannose-6-phosphate modification from being made. This defect ultimately leads to trafficking dysfunction in cells from these patients (Griffiths, 1995).

The lytic granule is an acid compartment with a pH of 5.5 (Henkart, 1985; Masson et al., 1990). Acidity of the granule is maintained by the activity of a vacuolar type H^+-ATPase and it appears that acidification is essential for CTL-effector function (Kataoka et al., 1994; Kataoka et al., 1997). Raising the granule pH using H^+-ATPase inhibitors such as concanamycin A leads to proteolytic degradation and loss of function of effector molecules such as perforin (Kataoka et al., 1994; Kataoka et al., 1997). The acid environment of the granule may also help to keep effector molecules such as perforin or the granzymes inactive and this may contribute to protecting the CTL from its own killing machinery (Darmon et al., 1999).

1.2.3 Granule Exocytosis

Once target recognition has occurred, a number of profound alterations occur in the cellular architecture of the CTL. The Golgi-apparatus, microtubule-organizing centres, and the granules reorient towards the sites of contact between the CTL and the target in a Ca^{2+}-dependent fashion (Anel et al., 1994; Atkinson et al., 1990; Geiger et al., 1982; Kupfer and Dennert, 1984; Kupfer et al., 1985; Kupfer et al., 1986; Yannelli

et al., 1986). It is thought that these architectural transformations ensure that exocytosis occurs unidirectionally from CTL to target, fostering both efficient killing of the target and protection to the CTL and surrounding tissues (Dennert et al., 1985).

The transit of granules within the CTL occurs along microtubules through a kinesin-dependent mechanism (Burkhardt et al., 1993). Kinesin activity, in turn, is regulated by the phosphorylation of kinesin-associated proteins (McIlvain et al., 1994) thereby linking exocytosis with the phosphorylation events observed following target cell recognition (Anel et al., 1996; Anel et al., 1994; Rosato et al., 1994).

The entire process of granule exocytosis is not clearly defined but it is probable that secretion parallels the mechanism that has been described for mast cells. Mast cell secretion depends upon the function of a number of small GTPases of the Rac and Rho family (Norman et al., 1996; Price et al., 1994). Rac and Rho regulate F-actin polymerization which in turn regulates the organization of actin filaments that are critical for cytoskeletal reorganization of mast cell granules (Norman et al., 1996; Price et al., 1994). Blocking Rac or Rho activity with an irreversibly binding GTP analogue (GTP-γ-S), or inhibiting actin polymerization with phalloidin prevents actin polymerization but does not completely inhibit secretion (Norman et al., 1996). Hence Rac and Rho are likely important for positioning mast cell granules at the plasma membrane but do not play a direct role in exocytosis per se. Rho proteins have been identified in CTLs (Lang et al., 1992) and the expression of Rac 2 is markedly upregulated following mitogenic stimulation of lymphocytes (Reibel et al., 1991) suggesting that CTL may utilize an exocytosis mechanism similar to mast cells.

1.3 CTL-Derived Granule Proteins Involved in Target Cell Death

As mentioned in **section 1.2.2**, the granules of CTL contain a number of proteins (see **Tables 1.2-1** and **1.2-2**). Although not all of these proteins have

been associated with target cell death, a select few have been directly implicated in the biochemical and morphological features characteristic of dying cells. Perforin, a pore-forming protein has long been linked with target cell cytolysis while members of the granzyme family of enzymes have been connected with a number of cytotoxic consequences. Granzymes A, B, H and K are the only granzymes expressed in activated human CTL and of these proteins, granzymes A and B are the most abundantly expressed. It is likely for these reasons that granzymes A and B are the best understood of the granzymes both in terms of structure/function relationships and their biological actions. Perforin and the human granzymes are summarized in the following sections.

1.3.1 Perforin

Perforin was one of the first biological molecules to be directly linked with CTL-mediated killing and for some time was thought to be the sole mediator of target cell lysis. Although it is now recognized that many other molecules participate in the killing process, the necessity for perforin in CTL-dependent death remains unquestioned. The following sections outline current views on the structure and role of perforin in CTL-dependent killing.

1.3.1.1 Perforin Isolation, Localization, and Structure

Perforin (cytolysin, pore-forming protein or ninth component (C9) of complement related protein) was originally isolated from CTL, NK cells and large granular lymphocytes as the protein responsible for causing lesions in target cell membranes (Liu et al., 1986; Masson and Tschopp, 1985; Podack et al., 1985; Young et al., 1986; Zalman et al., 1986; Zalman et al., 1985). Perforin is a 66-75 kDa, hydrophobic, single-chain polypeptide with homology to the C9 protein of complement. In the lytic granules where the pH is estimated to be 5.5 (Henkart et al., 1987), perforin is bound with chondroitin sulphate proteoglycans (Tschopp and Masson, 1987) and

calreticulin (Andrin et al., 1998). Collectively, the low pH of the granule as well as binding to the proteoglycans and calreticulin are all thought to prevent perforin from doing harm to the cytotoxic lymphocyte in which the granule resides (Andrin et al., 1998; Kataoka et al., 1994; Kataoka et al., 1997; Masson et al., 1990; Tschopp and Nabholz, 1987). Following exocytosis, the neutral pH of the intermembrane space causes the release of proteoglycans (Schmidt et al., 1985), thereby freeing perforin.

Interestingly, calreticulin, traditionally considered an ER resident protein because of a C-terminal KDEL[1] sequence, has been localized to cytolytic granules following T cell activation (Burns et al., 1994; Dupuis et al., 1993). Calreticulin has both Ca^{2+}-binding activity and chaperone functions (Michalak et al., 1999). Since perforin co-purifies with calreticulin, it is possible that calreticulin's role in the granule is that of chaperone. Alternatively, since CTL degranulation depends upon Ca^{2+} and since perforin function absolutely requires Ca^{2+} (see **section 1.3.1.2**), calreticulin may simply sequester Ca^{2+} within the granule to block perforin function. Recently, a report has proposed that calreticulin may actually play an extra-granular role in perforin-mediated osmotic lysis by stabilizing membranes to prevent poly-perforin channel formation through a mechanism independent of calreticulin's ability to bind Ca^{2+} or to directly interact with perforin (Fraser et al., 2000).

1.3.1.2 Perforin Function and its Role in Cell Lysis

Once released into the neutral environment of the intermembrane space and in the presence of Ca^{2+}, perforin undergoes a dramatic conformational change. The normally hydrophilic protein exposes hydrophobic domains in the presence of Ca^{2+} allowing perforin monomers to insert into the target cell membrane (Blumenthal et al., 1984; Ishiura et al., 1990; Young et al., 1987; Yue et al., 1987). It appears that the

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[1] Single letter amino acid abbreviations used.

composition of the target cell membrane may affect the ability of perforin monomers to insert (Rochel and Cowan, 1997). In this regard, it is interesting that insertion has been proposed to require binding of perforin to phosphorylcholine molecules on the target cell surface (Tschopp et al., 1989). Once inserted into the membrane, monomeric perforin is thought to recruit other perforin monomers to form stable non-selective channels with a diameter of 15-16 nm (Young et al., 1986). These pores are visible by electron microscopy (EM) both in target cells and liposomes to which purified perforin has been added (Blumenthal et al., 1984; Dennert and Podack, 1983; Dourmashkin et al., 1980; Podack and Dennert, 1983). Such EM visible pores are estimated to require the insertion of between 10 and 20 perforin monomers although it has been reported that only 3-4 monomers are necessary to form a functional channel (Young et al., 1986). Despite the dogma associated with target cell lysis by large poly-perforin channels, it is likely that the smaller pores are the more physiologically relevant. The small pores probably cause osmotic lysis following the perturbation of membrane permeability rather than outright destruction of the outer membrane (Liu et al., 1995a; Liu et al., 1995b). Supporting experimental evidence comes from studies in which it was shown that the amino-terminal 22 residues of perforin exhibit negative cooperativity, thereby limiting the number of perforin monomers that can participate in channel formation to 3-5 (Rochel and Cowan, 1996).

By itself, perforin is able to induce the lysis of a number of cell types and this led to the hypothesis that perforin alone accounted for CTL-induced cell death. This notion was supported by studies with perforin knockout mice showing the requirement for perforin during granule-mediated cytotoxicity (Kagi et al., 1994; Kagi et al., 1994; Kojima et al., 1994; Lowin et al., 1994). However, perforin-mediated cell lysis cannot explain the DNA fragmentation detectable prior to membrane damage during CTL-mediated attack (Duke et al., 1983; Duke et al., 1989). Target cell DNA fragmentation

suggests that perforin alone cannot be responsible for all of the morphological features observed during CTL-mediated killing and implies the involvement of additional proteins.

Perforin is absolutely required for granule-dependent killing and it is widely hypothesized that the role of perforin is to facilitate entry of the granzymes through the formation of poly-perforin channels. Nevertheless, recent evidence now suggests that the granzymes may gain entry to the cell independently of perforin through receptor-mediated endocytosis (Motyka et al., 2000; Pinkoski et al., 1998). Both granzymes B and H have been shown to accumulate into cytoplasmic vesicles of unknown identity in the absence of perforin (Edwards et al., 1999; Pinkoski et al., 1998). However, the granzymes remain trapped in these vesicles unless perforin is also present implying a role for perforin in mediating vesicle breakdown to effect granzyme release to the cytoplasm (Pinkoski et al., 1998). The small physiological pores that perforin likely forms would not allow the passage of proteins but would permit ion fluxes across the plasma membrane (and perhaps other membranes) which would be more consistent with a signaling role for perforin rather than as a direct mediator of osmolytic damage. In addition, perforin has recently been shown to facilitate the translocation of granzymes to the nucleus hinting at a further indirect but necessary role for perforin in the nuclear events of CTL-mediated killing (Blink et al., 1999). While the importance of perforin in CTL-mediated killing has never been questioned, the exact nature of its involvement in cell death remains to be determined.

1.3.2 Granzyme B

There is now overwhelming evidence, both circumstantial and concrete, that perforin alone cannot cause all of the characteristics of granule-mediated cell death. The granzymes have been implicated as effectors of many of the biochemical alterations that occur within the target cell. In humans, granzymes

A and B are the most abundantly expressed following T_C cell activation. Of these two proteins, granzyme B has been the most characterized largely based on its clear and direct roles in proteolysis of known target cell proteins and the morphological consequences of these cleavage events. Granzyme B is examined in the next subsections.

1.3.2.1 Granzyme B Synthesis and Granule Localization

Granzyme B is synthesized as a 29 kDa zymogen but following synthesis, it is differentially glycosylated on two Asn residues in the ER and Golgi to generate species of 29-33 kDa (Caputo and Bleackley, unpublished). The added sugars include mannose-6-phosphate residues which, as with granzyme A, are important for targeting granzyme B to cytolytic granules although other targeting mechanisms likely exist (Griffiths and Isaaz, 1993). The zymogen form of granzyme B requires removal of an amino-terminal dipeptide by dipeptidyl peptidase I to become activated (McGuire et al., 1990; Smyth et al., 1995). The importance of this amino-terminal cleavage event has been illustrated experimentally by expressing recombinant granzyme B either with or without the amino-terminal dipeptide (Caputo et al., 1993). Granzyme B expressed with the dipeptide intact was unable to hydrolyze a peptide thiobenzyl ester substrate while granzyme B lacking the dipeptide was enzymatically active. Further, it has been shown that CTL-effector function is impaired in response to inhibition of dipeptidyl peptidase I activity (Thiele et al., 1997). It is possible that the necessity for removal of the activation dipeptide in the granule may help to ensure that improperly targeted granzyme does not wreak intracellular havoc. Further, the low pH of the granule and the fact that granzymes are bound to proteoglycans (Peters et al., 1991) likely participate to ensure that the granzymes are directed towards the target cell rather than the CTL.

1.3.2.2 Granzyme B Structure and Substrate Specificity

Granzyme B is a serine proteinase. Until recently, there were no X-ray crystal or NMR structures available for granzyme B. Nevertheless, granzyme B had been modeled based on sequence alignment with other serine proteinases (Murphy et al., 1988). The model showed that granzyme B fit the typical structure of chymotrypsin-like serine proteinases. Granzyme B was therefore predicted to consist of two β-barrel domains separated by a groove in which the residues making up the substrate binding site and the catalytic triad are found (Murphy et al., 1988). The model also placed an arginine residue at position 226 in the specificity pocket, thereby predicting a substrate preference for aspartate or glutamate residues in the P_1 position[2] (Murphy et al., 1988). Granzyme B was subsequently shown to have Aspase activity confirming this predicted specificity (Odake et al., 1991; Poe et al., 1991). The requirement for cleavage following aspartate residues was reinforced by studies examining the hydrolysis of thiobenzyl ester substrates following mutation of the active site Arg 226 in the specificity pocket of granzyme B (Caputo et al., 1994; Caputo et al., 1999). Mutation to glycine or glutatmate converted the specificity to cleavage following hydrophobic or basic residues respectively. During the preparation of this thesis, the X-ray crystal structure of granzyme B in complex with a peptide inhibitor was published (Waugh et al., 2000). It has confirmed many of the structural and functional predictions outlined above.

The use of a combinatorial peptide library to screen for the ideal substrate cleavage site for granzyme B predicted a preference for cleavage at sites with the sequence (I/L/V)EXD↓ (where cleavage occurs carboxyl to the D residue) (Thornberry et al., 1997). This substrate specificity was further extended to include residues

flanking the tetrapeptide cleavage site: (I/V)(G/M/E)XD↓XG (Harris et al., 1998). Cleavage on the carboxyl side of aspartate residues is a unique specificity among mammalian serine proteinases but as we shall see in **section 1.5**, it is a strikingly similar requirement to another family of proteinases that are intimately connected with cell death, the caspases. Caspases require cleavage following aspartate residues to become activated (**section 1.5.1.2**) and the fact many of the caspases contain cleavage sites recognized by granzyme B suggests that granzyme B can directly activate the caspases within targets (more will be said on the involvement of granzyme B in caspase activation in **section 1.7.2**).

1.3.2.3 Evidence for Involvement of Granzyme B in CTL-Mediated Killing

In the early 1980s it was recognized that target cell lysis by CTL was preceded by fragmentation of cellular DNA in a fashion resembling apoptosis (apoptosis is discussed in **section 1.4**) (Duke et al., 1983; Duke et al., 1986; Russell et al., 1980). However, it was another decade before the proteins responsible for this CTL-dependent DNA fragmentation were isolated and characterized. Using purified rat NK cell granules and a whole cell assay for measuring DNA fragmentation, three 'fragmentin' activities were identified: fragmentin-1 (now recognized as granzyme A), fragmentin-2 (granzyme B) and fragmentin-3 (granzyme K) (also see **Table 1.2-2**) (Shi et al., 1992; Shi et al., 1992). Fragmentin-2 (granzyme B) proved to be the most rapidly acting of the fragmentins. This fragmenting activity did not require protein synthesis although it did depend absolutely upon the presence of perforin (Shi et al., 1992). An alternative approach involving transfection of granzyme A or B into rat basophilic leukemia cells (RBLs) either alone or in combination with perforin led to a similar

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[2] Here, the standard proteinase nomenclature has been used where P_1 denotes the position within the enzyme that binds carboxyl to the scissile bond of the substrate. The corresponding amino acid within the substrate is referred to as S_1 and proteolysis occurs on the carboxyl side of this residue.

conclusion for the role of granzyme B (Nakajima and Henkart, 1994; Shiver and Henkart, 1991; Shiver et al., 1992). RBLs are mast-like cells that contain granules. When induced to undergo degranulation through binding to IgE-coated targets, RBLs only showed tumour-directed DNA fragmentation and lysis when both granzyme B and perforin were co-transfected.

Further evidence for the involvement of granzyme B in target cell lysis comes from studies with granzyme B knockout mice. Mice with a homozygous null mutation in the granzyme B gene develop normally and have normal hematopoeisis and lymphopoeisis (Heusel et al., 1994). However, CTL or NK cells derived from these mice are defective for early induction of target cell DNA fragmentation although with longer incubation times, DNA fragmentation is still observed (Heusel et al., 1994). Granzyme B null CTL are still able to cause target cell membrane damage albeit with reduced efficiency (Heusel et al., 1994).

1.3.3 The Involvement of Other Granzymes in CTL-Mediated Killing

The kinetic nature of DNA fragmentation and the partial inhibition of membrane damage in the granzyme B$^{-/-}$ mice suggest that other granzymes may also participate in target disruption. In fact, granzyme A has been shown to account for much of the late DNA fragmentation observed during CTL-mediated killing through a mechanism that can be blocked by protein synthesis inhibitors such as cycloheximide (at least in rats (Shi et al., 1992)) as well as by serine protease inhibitors (Nakajima and Henkart, 1994; Poe et al., 1991; Redelman and Hudig, 1983). Indeed, granzyme A has recently been shown to initiate an apoptotic pathway independent of caspases leading to DNA fragmentation resulting from single, rather than double strand breaks (Beresford et al., 1999; Shresta et al., 1999). These single strand breaks cannot be visualized by

agarose gel electrophoresis or standard [^{3}H]thymidine release assays (Beresford et al., 1999).

The roles of other granzymes are poorly defined. Nonetheless, recent studies have shown that human granzyme K can act in conjunction with perforin to cause cell death (MacDonald et al., 1999) and may also possess a small but detectable DNA fragmenting ability (Shresta et al., 1997). In addition, human granzyme H was recently shown to enter cells through a receptor-dependent process and to accumulate into intracellular vesicles reminiscent of the mechanism utilized by granzyme B implying that granzyme H may have hitherto unknown intracellular function(s) (Edwards et al., 1999). Despite the involvement of other granzymes in target cell death the kinetic impairment of rapid DNA fragmentation with granzyme B$^{-/-}$ CTL underscores the importance of this granzyme for rapid and efficient CTL-dependent killing in vivo. A more detailed explanation of granzyme B's role in target cell death awaits **section 1.7.2.**

1.4 Classification of Cell Death

There are primarily two types of cell death that have been thoroughly -although by no means completely- characterized; they are apoptosis and necrosis. Both types have been observed during CTL-mediated killing although the former is considered the primary mechanism utilized by CTL to kill their targets (for review see Atkinson and Bleackley, 1995; Darmon et al., 1999; Rathmell and Thompson, 1999; Shresta et al., 1998; Trapani et al., 2000). Apoptosis and necrosis are outlined in the next sections.

1.4.1 Apoptosis

The word apoptosis is of Greek origin and describes leaves falling from trees or petals from flowers. The term was originally coined by Kerr, Wyllie and Currie in the

early 70s to describe an as yet uncharacterized type of cell death occurring within animal cell populations (Kerr et al., 1972). In its infancy, apoptosis was understood primarily in a morphological context. Nonetheless, the last 30 years have fostered tremendous understanding of the biochemical mechanisms underlying the morphology of apoptosis. This understanding is far from complete but the emerging themes indicate that the process is ubiquitous within tissues of multicellular organisms, that most, if not all cells are capable of dying by apoptosis and that apoptosis is critical for development, homeostasis, and the normal functions of the immune system. Cells that escape apoptosis at the end of their functional lifespans give rise to tumourigenesis or autoimmune diseases. In contrast, enhanced apoptosis leads to acute pathologies such as ischemia-reperfusion injury, infection by toxin-producing microorganisms as well as chronic diseases such as neurodegenerative disorders and AIDS. Consequently, apoptosis functions to balance proliferation and to combat damage acquired by individual cells within populations.

Apoptosis involves the removal of cells from within tissues in a deliberate and systematic manner (Duvall and Wyllie, 1985; Kerr et al., 1972). Dying cells shrink and detach from neighbouring cells (**Table 1.4-1**). Both the cytoplasm and nucleus become markedly condensed. The nucleolus and nuclear envelope break apart as the underlying structures are disassembled (Duvall and Wyllie, 1985; Kerr et al., 1972). In addition, the chromatin condenses and the plasma membrane forms convoluted invaginations and protrusions called blebs that can pinch off to form apoptotic bodies containing both condensed and morphologically normal organelles.

In addition to the morphological features observed, a number of biochemical events also occur. Probably the most notable biochemical marker is the cleavage of condensed chromatin into internucleosomal fragments of about 180-200 base pairs (Skalka et al., 1976; Wyllie, 1980; Wyllie et al., 1984). Such DNA fragments give rise to

Table 1.4-1 Comparison of Apoptosis versus Necrosis[1]

Apoptosis	Primary Necrosis
Physiological or pathological (subnecrotic damage)	Accidental, always pathological
Tightly regulated process	Unregulated or poorly regulated
Plasma membranes remain nearly intact until late	Plasma membrane destroyed early
Phagocytic elimination (either by professional phagocytic cells such as macrophage or dendritic cells or by non-professional neighbouring cells)	No specific removal of necrotic cells observed
No leakage of cell contents: little or no inflammation	Cell contents leak into surrounding tissue: inflammation
Participation of cellular enzymes leading to characteristic biochemical and morphological features: chromatin condensation, nuclear breakdown, DNA fragmentation into ~200 base pair oligonucleosomal sized fragments, selective protein cleavage (mediated by caspases), plasma membrane alterations (loss of membrane asymmetry precedes loss of integrity), cell shrinkage, no mitochondrial swelling	Biochemical and morphological features include: swelling of entire cytoplasm and celluar organelles; mitochondria in particular, no regular pattern of DNA fragmentation or protein degradation
	Secondary Necrosis
	Dying apoptotic cells that are not removed by phagocytosis undergo secondary necrosis

[1]Adapted from (Kroemer et al., 1998). Kroemer has noted that while many consider apoptosis and necrosis to be two separate courses of response, it is probably more correct to think of both processes as representing two extremes of the same continuum. When the death stimulus is 'gentler', apoptosis is favoured. For example, a toxin at low (subnecrotic) concentration may induce apoptosis while at high dose may cause necrosis. This is also illustrated by the fact that apoptotic cells in culture that cannot be properly phagocytosed undergo secondary necrosis.

a laddering effect by agarose gel electrophoresis and this characteristic is a hallmark of apoptosis. Additionally, the early manifestations of apoptosis involve phosphatidylserine molecules, which normally reside on the inner leaflet of the plasma membrane. Following an apoptotic stimulus, phosphatidylserine molecules flip to the outer leaflet of the plasma membrane (Castedo et al., 1996; Martin et al., 1996; Martin et al., 1995). Phosphatidylserine externalization is thought to arise as a result of both decreased activity of an ATP-dependent amino-phospholipid translocase and an increased activity of a Ca^{2+}-dependent lipid scramblase (Bratton et al., 1997; Verhoven et al., 1995). Phosphatidylserine exposure likely serves as a stimulus to macrophage and non-professional phagocytic cells that the apoptotic cell or apoptotic bodies should be phagocytosed (Fadok et al., 1998; Fadok et al., 1998). A number of receptors found on phagocytic cells have recently been proposed to bind externalized phosphatidylserine on apoptotic cells to mediate the phagocytic signal (Fadok et al., 1998; Fadok et al., 2000).

Other biochemical features characteristic of apoptosis include the loss of mitochondrial membrane potential ($\Delta\Psi_m$), the efflux of apoptogenic proteins including cytochrome c from the mitochondrial intermembrane space to the cytosol and the proteolytic activation of a family of cysteinyl aspartate-specific proteinases (caspases) that are thought to be responsible for many of the morphological features observed during apoptosis. Caspases will be considered in **section 1.5**, while mitochondrial events will be discussed in detail in **section 1.6**.

The complete apoptosis of a cell is a rapid process occurring within 24 hours and typically occurring over a much shorter period. Apoptosis mediated by CTLs, for example, can begin within 30 minutes or less following the addition of CTL to targets (Heibein and Bleackley, unpublished observations). Importantly, the membrane integrity of the apoptotic bodies remains and they are phagocytosed before there is

leakage of cytosolic contents into the surrounding tissues to induce an inflammatory response (Rathmell and Thompson, 1999).

While apoptosis has long been considered immunologically quiescent, emerging evidence suggests that dendritic cells can illicit an immune response by presenting processed peptides from phagocytosed apoptotic bodies, although the immunogenicity of these peptides appears to be very low (Albert et al., 1998; Bellone et al., 1997; Ronchetti et al., 1999; Sauter et al., 2000). Additionally, during apoptosis, pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β and IL-18 are processed and secreted (Hogquist et al., 1991).

1.4.2 Necrosis

In contrast with apoptosis, necrosis is a type of cell death typically occurring in large numbers of cells within a tissue in response to physiological trauma such as acute anoxia, a sudden shortage of nutrients, or extreme physical or chemical injury. Necrotic cells die in an indiscriminate fashion. In contrast with the cell shrinkage of apoptosis, necrotic cells and the organelles within lose the ability to regulate osmotic homeostasis and swell as they take up water (Rathmell and Thompson, 1999). Cell swelling ultimately leads to rupture of the plasma membrane with subsequent leakage of cellular contents into the surrounding tissue and, invariably, a consequent inflammatory response. Unlike the DNA laddering seen early within apoptotic cells, DNA fragmentation in necrotic cells is a late event occurring in a haphazard fashion through a mechanism dependent on serine proteinases (Bicknell and Cohen, 1995; Dong et al., 1997; Duvall and Wyllie, 1985). The result is the production of DNA fragments of varying size that appear as a smear by agarose gel electrophoresis. The overall view of necrosis is that the cells appear to fall apart rather than to be systematically disassembled as occurs with apoptosis.

1.5 Target Cell Proteins Involved in Cell Death

While many of the signals inducing a cell to die may come from exogenous sources, the process of cell death is now recognized to depend upon the activities of proteins within the cell itself. A decade ago, these proteins were largely unknown. However, the last 10 years have brought with them a remarkable understanding of the players involved in cell death and the mechanisms they employ. The next sections outline a number of these proteins and what is known about their functions.

1.5.1 Caspases

Some of the first biological molecules associated with cell death were members of a family of proteins now known as caspases (Alnemri et al., 1996). The following section offers an overview of the structures and functions of these proteins. A more detailed description of the specific roles of caspases in CTL-mediated cell death awaits **section 1.7**.

1.5.1.1 Caspase History and Organization

Caspases (cysteinyl aspartate-specific proteinases) play a critical role in the morphology of apoptosis and in some cases are essential for its induction (for review see Earnshaw et al., 1999; Nicholson, 1999; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). The first acknowledged caspase, interleukin-1β converting enzyme (ICE; caspase-1), was originally identified through its involvement in the processing of proIL-1β to the mature biologically active pro-inflammatory IL-1β molecule (Carretti et al., 1992; Thornberry et al., 1992). ICE was the first of a new family of cysteinyl proteinases defined by both structural features and by the absolute requirement for cleavage on the carboxyl side of aspartic acid residues (Earnshaw et al., 1999; Nicholson, 1999; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). At the time of its unearthing, ICE was shown to play a role in inflammation but

other functions of the protein were not known. Concomitant with the discovery of ICE, a gene called ced-3 was found in the nematode *C. elegans* that proved critical for all of the 131 programmed cell deaths observed throughout the development of the worm (Yuan and Horvitz, 1990). Cloning and sequencing of ced-3 showed it to be a homologue of ICE, hinting at an additional role for ICE in mammalian cell death (Xue et al., 1996; Yuan et al., 1993). Since the early 1990s, 10 additional human caspase family members have been identified and 14 are known to occur in mice (**Figure 1.5-1**). The caspases can be phylogenetically divided into two sub-families based on their relatedness to either ICE (caspase-1) or ced-3 (**Figure 1.5-1**). The caspases can be further subdivided based on their structural organization. Caspases-3, -6 and -7 contain short pro-domains while the rest of the caspases have long pro-domains. Additionally, the caspases may be organized based on their substrate specificities. As indicated above, caspases have an absolute requirement for aspartic acid in the P_1 position. However, the residues amino to the S_1 position (S_2, S_3, S_4 etc) also play a role in substrate binding. The optimal substrate recognition sequences for the caspases have been determined using a positional scanning substrate library (Rano et al., 1997; Thornberry et al., 1997). Based on these substrate specificities, caspase-1, -4, -5, and -13 fall into group I and have a preference for cleavage following the sequence (W/L)EHD. Group I caspases have been implicated in cytokine processing and inflammation. Group II caspases (caspase-2, -3 and -7) have the preferred cleavage motif DExD. Many proteins that are proteolytically cut during apoptosis are cleaved at DxxD sites confirming the role of caspases in cell death (Nicholson, 1999; Stroh and Schulze-Osthoff, 1998). Group II caspases are also referred to as effector caspases since these caspases are responsible for cleaving substrates downstream within the apoptotic program that are responsible for apoptotic morphologies. In contrast, group

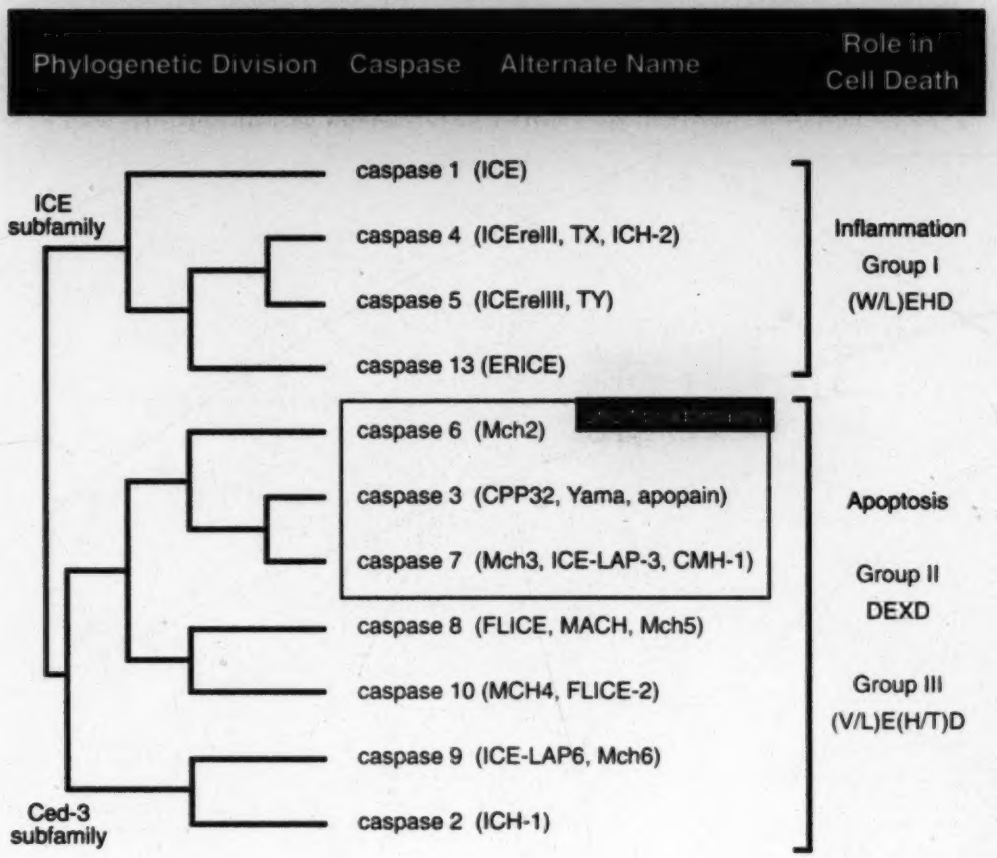


Figure 1.5-1 Caspase Phylogenetic Divisions and Substrate Specificities

Caspases can be subdivided based on how they fall into phylogenetic catagories (either into the ICE or the Ced-3 subfamilies), or based on proteolytic specificities (as indicated). Caspases can be additionally subdivided into three groups based on function: group I enzymes (blue) mediate cytokine maturation; the remainder of the caspases participate in apoptosis. Group II (red) caspases are the effectors of cell death while group III (green) members are initiator (activator) enzymes. The majority of caspases have long pro-domains (>10 kDa) except for caspases -3, -6, and -7 which have short peptide pro-domains (<30 amino acids). Adapted from (Nicholson, 1999).

III caspases (caspase-6, -8, -9 and -10) prefer branched chain aliphatic residues at the P_4 position ((V/L)E(T/H)D). These motifs are found at the maturation sites of most of the group II and group III caspases, hinting at a role for activation of these caspases by caspases themselves. Indeed such organization is consistent with the known roles of the group III caspases as upstream activators of the group II caspases (Earnshaw et al., 1999; Nicholson, 1999; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). Since group III caspases are frequently responsible for activating caspases downstream of the initial apoptotic signal, they are often referred to as initiator caspases.

The link between caspases and apoptosis has been solidified through studies utilizing inhibitors (outlined in **section 1.5.1.5**) as well as caspase knockout mice. Knockout mice are currently available for caspases 1, 2, 3, 8, 9, and 11 (**Table 1.5-1**). Generally speaking, caspase disruption leads to the abrogation of apoptosis in a variety of tissues and stages of development (reviewed in Colussi and Kumar, 1999; Earnshaw et al., 1999; Zheng et al., 1999). However, the exact nature of these disruptions depends upon the caspase knockout in question indicating that caspases are not entirely redundant in cell death and that multiple caspase activation pathways are likely to exist. For example, caspase-1 knockout mice are developmentally normal. The most profound defect observed in these mice is an absence of processing and secretion of IL-1β consistent with caspase-1's role in inflammation. Nevertheless, some resistance to Fas-mediated killing is observed in thymocytes isolated from caspase-1 knockout mice (Kuida et al., 1995; Li et al., 1997) (Fas-mediated killing is described in **section 1.7.1**).

Caspase-3 knockouts on the other hand, exhibit a more severe phenotype. Caspase-3$^{-/-}$ mice survive birth but suffer from high perinatal mortality as a result of developmental defects in the brain correlating with decreased apoptosis

Table 1.5-1 Effects of Caspase Gene Disruption[1]

Caspase	Development	Apoptotic Dysregulation	References
Caspase-1	normal	Fas killing (thymocytes)	(Kuida et al., 1995; Li et al., 1997)
Caspase-2	normal	germ cells	(Bergeron et al., 1998)
Caspase-3	perinatally lethal*	Neuroepithelial progenitors; Delayed or absent morphological changes; Absence of DNA fragmentation	(Kuida et al., 1996; Woo et al., 1998)
Caspase-8	embryonic lethal	Death receptor pathways (Fas/TNF) in EFs; Neuroepithelial progenitors; Mitochondrial pathways (dexamethasone, etoposide, staurosporine, γ-irradiation) in thymocytes	(Varfolomeev et al., 1998)
Caspase-9	embryonic lethal**	Defective brain development; ES and EF resistant to apoptosis; Thymocytes resistant to dexamethasone, and γ-irradiation but not Fas	(Hakem et al., 1998; Kuida et al., 1998)
Caspase-11	normal	resistant to endotoxic shock induced by lipopolysaccharide; Production of IL-1α and IL-1β blocked ; Thymocytes resistant to apoptosis induced by caspase-1 overexpression	(Wang et al., 1998)

[1]Adapted from (Zheng et al., 1999).

*Depends on genetic background (see (Zheng et al., 1999))

**<2% survive birth but those that do develop normally

ES cells, embryonic stem cells

EFs, embryonic fibroblasts

(Kuida et al., 1996; Woo et al., 1998). Many other tissues, including the thymus, heart and liver appear normal at birth and thymocytes appear to undergo apoptosis normally in response to anti-Fas, dexamethasone, staurosporine and γ-irradiation (Kuida et al., 1996; Woo et al., 1998). In contrast, neutrophils and activated T cells seem to require caspase-3 for apoptosis (Woo et al., 1998). Importantly, while death does occur in cells cultured from a number of tissues of caspase-3 null mice, the cell death observed is characterized by an absence of DNA fragmentation underscoring the role of caspase-3 in this process (Woo et al., 1998).

1.5.1.2 Caspase Activation

Caspases exist within cells as immature pro-enzymes that must undergo proteolytic cleavage at a minimum of two sites containing aspartate residues to become activated (**Figure 1.5-2**). Processing appears to follow a specific pattern where the initial cleavage event follows an aspartate residue separating the large and small subunits followed by a second cleavage event to remove the pro-domain (Earnshaw et al., 1999; Nicholson, 1999). Pro-caspases have a low level of intrinsic activity and it is now recognized that when brought into close proximity and in the absence of other processing, pro-caspases can transactivate themselves (Earnshaw et al., 1999).

It is believed that two general mechanisms exist for this autoprocessing. The first, called scaffold-mediated activation (Earnshaw et al., 1999), typically applies to the processing of initiator (group III) caspases which lie upstream within caspase activation cascades. The activation of caspase-8, for example, involves the recruitment of pro-caspase-8 to an adapter molecule, FADD/MORT1, that tethers the pro-caspase to the Fas (CD95/APO-1) death receptor following binding of Fas ligand on the cell surface (Boldin et al., 1996; Boldin et al., 1995; Chinnaiyan et al., 1995; Chinnaiyan et al., 1996; Medema et al., 1997; Muzio et al., 1996) (Fas-mediated apoptosis is described

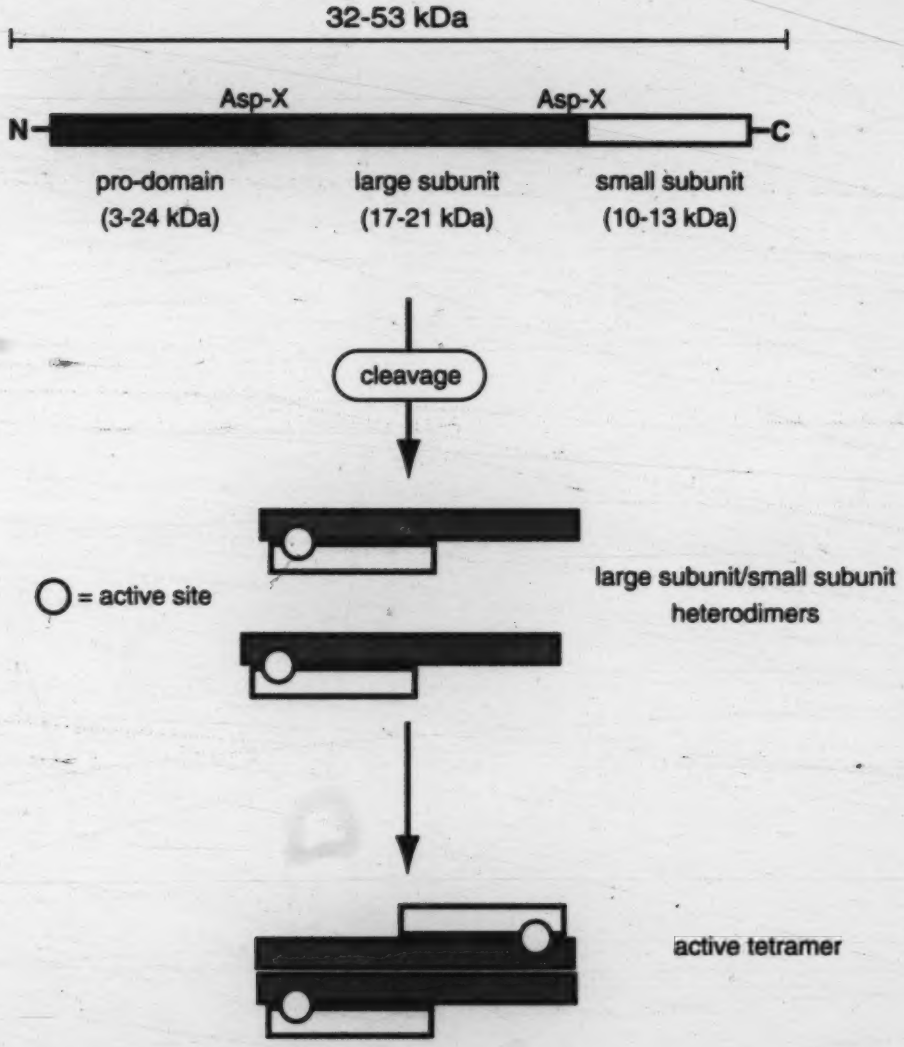


Figure 1.5-2 Caspase Pro-Enzyme Organization and Activation

Caspases require proteolytic cleavage following aspartate residues to liberate the small and large subunits and to release the pro-domain. Following cleavage, the large and small subunits join to form a heterodimer containing the active site (hollow circle). Two heterodimers then join to form the active tetrameric enzyme with the active sites facing away from each other (see text).

in more detail in **section 1.7.1**). This process creates a microenvironment with a high local concentration of caspase-8 zymogens, allowing transactivation to efficiently generate active caspase-8 molecules [(Martin et al., 1998; Muzio et al., 1998; Yang et al., 1998). The processing of pro-caspase-9 follows a similar pattern where its inclusion in a complex with Apaf-1, cytochrome c and dATP or ATP allows for transactivation yielding free active caspase-9 (Hu et al., 1999; Li et al., 1997; Pan et al., 1998; Saleh et al., 1999; Srinivasula et al., 1998; Zou et al., 1999). Collectively, the available data indicates that the scaffold-mediated activation of initiator (group III) caspases can result in the initiation of caspase cascades.

The effector (group II) caspases can also undergo autocatalytic activation at least in vitro, however there is currently no evidence supporting such an activation mechanism in biological systems (Earnshaw et al., 1999). Rather than utilizing scaffold-dependent processing, it appears that group II caspases are efficiently activated largely by other caspases or by the CTL granule product, granzyme B. For example, caspase-8 efficiently cleaves pro-caspases 3, 4, 7, and 9 in vitro (Muzio et al., 1997; Srinivasula et al., 1996; Takahashi et al., 1997) and caspase-9 can activate caspase-3 (caspase-9-mediated activation of caspase-3 is discussed in more detail in section 1.6.2).

1.5.1.3 Caspase Structure

The X-ray crystal structures available for caspase-1 and -3 indicate that both active caspases exist as tetramers containing two heterodimers positioned in a head to tail arrangement (Mittl et al., 1997; Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994). Each heterodimer is composed of a small and large subunit derived following cleavage at internal aspartate residues. The large and small subunits come together to form the active sites of these enzymes which utilize a characteristic

cysteine protease mechanism involving a catalytic diad of cysteine and histidine (Rotonda et al., 1996; Walker et al., 1994; Wilson et al., 1994). In the active tetramer, the active sites formed by the (small subunit:large subunit) heterodimers are found on opposite ends of the molecule. Despite the occurrence of two active sites in the tetramer, there is currently no evidence supporting cooperativity or allosteric regulation at these sites (Nicholson, 1999).

1.5.1.4 Caspase Substrates and the Effects of Caspase Activation

A complete list of the known caspase substrates is beyond the scope of this thesis (for more complete reviews of caspase substrates see Earnshaw et al., 1999; Nicholson, 1999; Stroh and Schulze-Osthoff, 1998). However, a select group of substrates illustrates the variety of cellular functions disrupted during apoptosis (**Table 1.5-2**). Caspases mainly function to switch off protective and/or homeostatic mechanisms and to switch on degradative pathways that lead to the destruction of the cell. Of the substrates listed in **Table 1.5-2**, a number have been intimately connected with the morphologies typical of apoptosis. For example, gelsolin is a protein involved in Ca^{2+}-dependent regulation of actin polymerization and is important for maintenance of the cytoskeleton (Kwiatkowski, 1999). Gelsolin is cleaved by caspase-3 during apoptosis thereby relieving gelsolin from Ca^{2+}-dependent regulation. This cleavage event is thought to contribute to the cytoskeletal rearrangements observed during apoptosis (Kothakota et al., 1997). Another important caspase-3 substrate is the inhibitor of caspase-activated DNase (ICAD) that is largely responsible for the hallmark DNA fragmentation of apoptosis (Enari et al., 1998; Wolfe et al., 1999). ICAD normally inhibits the DNase activity of the caspase-activated DNase (CAD) but caspase-3-dependent cleavage of ICAD removes this block and allows CAD-dependent DNA fragmentation to occur (Enari et al., 1998; Wolfe et al., 1999).

Table 1.5-2 Selected Caspase Substrates[1]

Substrate	Cleavage Site	Caspase Responsible	Proposed Effect of Cleavage	References
Gelsolin	DQTD↓G	3	Calcium-insensitive actin cleavage	(Kamada et al., 1998; Kothakota et al., 1997)
Lamin A	VEID↓N	6	Nuclear lamina disassembly	(Lazebnik et al., 1993; Oberhammer et al., 1994; Rao et al., 1996; Takahashi et al., 1996)
NuMa	?	3,6	Nuclear shape change	(Casiano et al., 1996; Hirata et al., 1998)
Focal adhesin kinase	DQTD↓S VSWD↓S	3,7 6	Loss of paxillin binding; ? translocation to nucleus; ↓activity	(Crouch et al., 1996; Gervais et al., 1998; Wen et al., 1997)
Ras GTPase-activating protein	DTVD↓G	3	Inactivation of survival signaling from extracellular receptors	(Wen et al., 1998; Widmann et al., 1998)
ICAD	DEPD↓S	3	Liberates active CAD endonuclease	(Enari et al., 1998)

[1]Adapetd from (Earnshaw et al., 1999). Note: only a select group of substrates shown here. For a more complete listing, see (Earnshaw et al., 1999)

Added to the increasingly bewildering array of cellular proteins cleaved by caspases, are caspases themselves (Earnshaw et al., 1999; Nicholson, 1999). In fact, caspases are only activated by other caspases (granzyme B-mediated caspase activation being the notable exception). Such a limited group of activators indicates the tight control under which these proteins are normally held. However, once initiated, caspase activation resembles more of a web rather than a linear sequence (see **Figure 1.6-2**) and such cross-talk may ensure that following caspase activation, the cell is irrevocably committed to die.

It is of note that the biological significance of many of the cleavage events occurring during apoptosis is not completely understood (Earnshaw et al., 1999). Moreover it is important to recognize that not all of the known caspase substrates are cleaved in all cell types in response to the same apoptotic stimuli nor indeed even within a single cell type in response to different apoptotic stimuli (Earnshaw et al., 1999). This heterogeneity likely reflects differential activation of caspases within differing cell types as well as differences between cells in the accessibility of substrates.

1.5.1.5 Caspase Inhibitors

It has already been mentioned that caspases have an absolute requirement for cleavage following aspartate residues. Additionally, the residues in the P_2-P_4 positions are important for determining substrate specificity. The known requirements for substrate recognition by caspases coupled with strategies that have proven effective for examining other cysteine proteases have allowed the design of potent synthetic peptide inhibitors that bind irreversibly to the active sites of the tetrameric enzymes (Garcia-Calvo et al., 1998). The most effective of these inhibitors contain peptide motifs corresponding the optimal substrate recognition sequences of the caspase of interest

(Garcia-Calvo et al., 1998). The peptides are generally coupled to aldehydes, nitriles or ketones to render them irreversible inhibitors but ketones have shown the most promise because of their longer half-lives in biological systems (Nicholson, 1999). Fluoromethyl ketones (fmks) have proven the most effective of the ketones since they appear to be the most cell permeable therefore requiring lower concentrations in biological systems to effect efficient caspase inhibition while avoiding issues of toxicity (Ekert et al., 1999). A recent study has shown that one of these fmk inhibitors, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) is an excellent competitive and irreversible inhibitor of caspases 1 to 10 (Garcia-Calvo et al., 1998). More specific inhibition of individual caspases can be achieved using peptides corresponding to the specific active site requirements of the caspase groups. For example, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (zDEVD-fmk) is a potent inhibitor of group II members caspase-3 and -7, which both prefer DEVD recognition motifs (Garcia-Calvo et al., 1998; Thornberry et al., 1997). However, zDEVD-fmk is not as potent an inhibitor of group II member caspase-2 which prefers DEHD motifs (Garcia-Calvo et al., 1998; Thornberry et al., 1997).

Although the mechanisms are incompletely understood, caspase inhibition also occurs through the action of biological inhibitors. For example the baculovirus p35 protein appears to be a broad-spectrum caspase inhibitor (Bump et al., 1995; Fisher et al., 1999; Xue and Horvitz, 1995) whereas the cowpox virus serpin, CrmA, is a potent inhibitor of group I and group III but not group II caspases (Garcia-Calvo et al., 1998). Members of another group of inhibitors, the inhibitor of apoptosis (IAP) superfamily, appear to be selective for group II caspases (Deveraux and Reed, 1999; Deveraux et al., 1998; Deveraux et al., 1997; Liston et al., 1996; Roy et al., 1997; Tamm et al., 1998). IAPs (XIAP, cIAP1 and cIAP2) were shown to block the proteolytic processing of pro-caspases -3, -6, and -7 by interfering with the cytochrome c-dependent

activation of pro-caspase-9 (Deveraux et al., 1998) (the role of cytochrome c in caspase activation is discussed in **section 1.6.2**). In addition, these IAPs have been shown to block the enzymatic activity of caspases -3 and -7 following their direct proteolytic processing through caspase-9 independent routes (Deveraux et al., 1997; Roy et al., 1997).

Inhibitors have proved invaluable tools for dissecting apoptotic pathways. More significantly, the shear variety of biological inhibitors emerging in nature points to the importance of regulating apoptosis in biological systems.

1.5.2 The Bcl-2 Family

It has become clear in the last decade that members of the Bcl-2 family of proteins play an integral role in regulating life/death decisions within cells. Bcl-2 and related proteins appear to supervise the cellular environment and act as sensors of stress in various cellular compartments. While an understanding of the mechanisms utilized by these proteins to monitor the cell is only now coming to light, it appears that once the decision to die has been made, a remarkably conserved series of events unfolds to attain the demise of the cell. The next section introduces a number of Bcl-2 family members and highlights current views on how they function in cell death. A more detailed description of the Bcl-2 family's role in CTL-mediated cell death awaits **sections 1.6** and **1.7**.

1.5.2.1 Bcl-2 is an Anti-Apoptotic Protein

Bcl-2 was originally identified as a gene from chromosome 18 that was constitutively over-expressed following a translocation event placing it under control of the immunoglobulin locus on chromosome 14 in human follicular lymphoma (Bakhshi et al., 1985; Cleary and Sklar, 1985; Cleary et al., 1986; Tsujimoto et al., 1985). Bcl-2 appears to function as an oncogene by providing signals allowing cells dependent upon growth factors to survive in the absence of these factors (Vaux et al., 1988).

Thus, Bcl-2 can influence cellular homeostasis by preventing cell death, thereby allowing cells to accumulate and become cancerous. In addition to the protection offered from growth factor withdrawal, Bcl-2 can also shield cells from a variety of other death stimuli including DNA damage, metabolic inhibitors, oncogene expression, glucocorticoid treatment and, in some cell types, Fas-induced cell death (Fas-mediated cell death is discussed in more detail in **section 1.7.1**) (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Korsmeyer et al., 1995; Reed, 1997; Reed et al., 1996). The anti-apoptotic effects of Bcl-2 in response to wide-ranging stimuli suggests that common mechanisms of cell death are utilized by these systems.

Bcl-2 has been localized to the endoplasmic reticulum, the nuclear envelope and to either the inner (Gotow et al., 2000; Hockenbery et al., 1990; Motoyama et al., 1998) or outer mitochondrial membranes (Krajewski et al., 1993; Nakai et al., 1993; Riparbelli et al., 1995). The protein is anchored through a C-terminal transmembrane domain to the membrane compartments where it is found (**Figure 1.5-3**). Removal of this motif either abrogates or diminishes Bcl-2's anti-apoptotic function (Hockenbery et al., 1993; Tanaka et al., 1993). However, the importance of this domain in targeting Bcl-2 to mitochondria has been demonstrated through its exchange for the transmembrane region of Mas-p70, a yeast mitochondrial outer membrane protein (Nguyen et al., 1993), or cytochrome b5 (Zhu et al., 1996). The alternative transmembrane motifs were sufficient to target Bcl-2 to mitochondria and for it to maintain full anti-apoptotic function.

1.5.2.2 Bcl-2 Defines a Conserved Family of Functionally Similar Proteins

Bcl-2 is now recognized as the prototyptic member of a family of genes whose products have been broadly divided into two categories based upon their prominent roles in apoptosis (**Table 1.5-3**). Some of the family members, including Bcl-2 and Bcl-

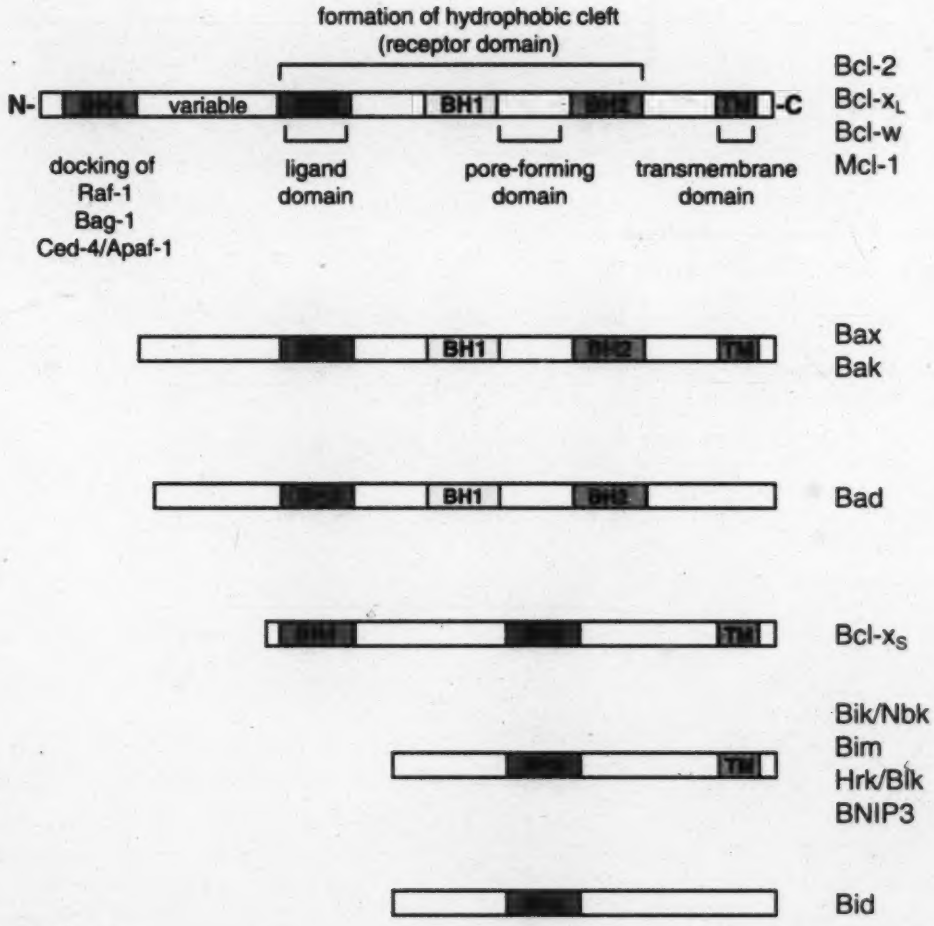


Figure 1.5-3 The Bcl-2 Family

The Bcl-2 family consists of both pro- (blue) and anti-apoptotic (red) members that are characterized by the presence of one or more Bcl-2 homology (BH) domains. Four types of BH domain have been described, BH1 to BH4, and various functions have been ascribed to each as indicated (also see **section 1.5.2**). A carboxy-terminal transmembrane (TM) domain is found in both pro- and anti-apoptotic proteins and is required for efficient function of these molecules.

x_L are anti-apoptotic while others such as Bid and Bax are pro-apoptotic. Both the pro- and anti-apoptotic members of the Bcl-2 family share one or more homologous domains referred to as Bcl-2 homology (BH) domains 1-4. (**Figure 1.5-3**) (for review see Adams and Cory, 1998; Chao and Korsmeyer, 1998; Kelekar and Thompson, 1998; Kroemer, 1997). The BH domains have a number of potential functions in regulating apoptosis that will be outlined in more detail in **section 1.6.4.2**. However, it is worth noting here that the only domain common to all family members, whether pro- or anti-apoptotic, is the BH3 domain while the BH4 domain is found exclusively in the anti-apoptotic members. The BH1 and BH2 domains have variable distributions.

1.5.2.3 Bid

The pro-apoptotic Bcl-2 family member Bid is a 26 kDa (human) protein first identified by its ability to heterodimerize with both Bcl-2 and another pro-apoptotic family member, Bax (Wang et al., 1996). Despite the absence of a transmembrane domain, Bid was found to have both cytosolic and membrane localizations and over-expression of the protein in the absence of other death stimuli was sufficient to induce apoptosis (Wang et al., 1996). Bid contains a single BH3 domain that is essential for its pro-apoptotic activity and its ability to heterodimerize with both Bcl-2 and Bax (Desagher et al., 1999; Wang et al., 1996). Interestingly, a Bid BH3 mutant that could still heterodimerize with Bcl-2 failed to induce apoptosis indicating that heterodimerization between Bid and Bcl-2 is not essential for Bid's pro-apoptotic activity (Wang et al., 1996). In contrast, heterodimerization between Bid and Bax was necessary to retain death-promoting activity. More recent studies have further elucidated the mechanism by which Bid stimulates apoptosis and as these mechanisms likely impinge upon CTL-dependent killing, this information will be disclosed in **sections 1.6 and 1.7.**

Table 1.5-3 Bcl-2 Family Members

Pro-apoptotic	References
Bcl-2	(Adams and Cory, 1998; Chao and Korsmeyer, 1998; Korsmeyer et al., 1995; Reed, 1997; Reed et al., 1996)
Bcl-X_L	(Boise et al., 1993; Boise and Thompson, 1997)
Bcl-w	(Gibson et al., 1996)
Mcl-1	(Kozopas et al., 1993; Zhou et al., 1997)
A-1/Bfl-1	(Choi et al., 1995; Lin et al., 1993)
Anti-apoptotic	
Bax	(Oltvai et al., 1993)
Bak	(Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995)
Bcl-X_S	(Boise et al., 1993; Boise and Thompson, 1997)
Bok/Mtd	(Hsu et al., 1997; Inohara et al., 1998)
Bad	(Yang et al., 1995)
Nbk/Bik	(Boyd et al., 1995; Han et al., 1996)
Bid	(Wang et al., 1996)
Bim	(O'Connor et al., 1998)
Hrk	(Inohara et al., 1997)
Blk	(Hegde et al., 1998)
BNIP3 (formerly NIP3)	(Chen et al., 1999)

1.5.2.4 Bax

Another pro-apoptotic member of the Bcl-2 family, Bax, is a 21 kDa protein that was first identified through its ability to heterodimerize with Bcl-2 (Oltvai et al., 1993). Bax is found in the cytosol as well as peripherally associated with membranes (Goping et al., 1998) and similarly to Bid, its over-expression accelerates apoptosis induced by IL-3 withdrawal from an IL-3-dependent cell line (Oltvai et al., 1993). Bax contains BH1, BH2 and BH3 domains as well as a transmembrane domain (**Figure 1.5-3**) that is normally sequestered but becomes exposed following an apoptotic stimulus allowing Bax to insert into the mitochondrial outer membrane (Goping et al., 1998). The insertion mechanism probably involves the recruitment of Bax through BH3-domain-dependent heterodimerization with mitochondrially-associated Bid, thereby allowing the transmembrane domain of Bax to insert (Desagher et al., 1999; Eskes et al., 2000; Goping et al., 1998). Both the conformational change in Bax exposing the transmembrane domain and its translocation from the cytosol to mitochondria is prevented by Bcl-2 and this ultimately prevents caspase activation and apoptosis (Desagher et al., 1999; Murphy et al., 2000; Murphy et al., 1999; Murphy et al., 2000). The data presented above provide an excellent example of the antagonistic relationships between the Bcl-2 family members and their roles in regulating life/death decisions.

1.5.2.5 The Functions of Bcl-2 Family Members Based on Studies Using Knockout Mice

During embryogenesis, Bcl-2 is widely expressed but expression levels drop and become more restricted postnatally (Merry et al., 1994). Bcl-2$^{-/-}$ mice are born viable but the majority die of renal failure within a few weeks post parturition (Kamada et al., 1995; Nakayama et al., 1994; Veis et al., 1993). In addition, despite normal neuronal development, there is massive apoptosis in the spleen and thymus a few

weeks after birth. (Nakayama et al., 1994; Veis et al., 1993). Those mice that do survive turn gray within 5 to 6 weeks post parturition reflecting a decrease in melanocyte survival (Kamada et al., 1995; Nakayama et al., 1994; Veis et al., 1993). In these mice, both T and B lymphocyte lineages develop normally, however, they are unable to maintain homeostasis within the immune system (Nakayama et al., 1994; Veis et al., 1993). Therefore, Bcl-2 appears to be dispensable for neuronal and lymphocyte development but is essential for renal function and immune surveillance.

In contrast with Bcl-2$^{-/-}$ mice, Bcl-X_L deletion results in embryonic lethality by day 13 (Motoyama et al., 1995). Apoptosis is extensive throughout the brain and spinal cord in regions of post-mitotic, differentiating neurons thus pointing towards an essential role for Bcl-X_L in neuronal development. In the immune system, T cell maturation is unaffected by Bcl-X_L deletion whereas the number of mature B cells is markedly reduced (Ma et al., 1995; Motoyama et al., 1995). Interestingly, in normal mice, lymphocyte maturation is reciprocally regulated by Bcl-2 and Bcl-X_L expression levels (Chao and Korsmeyer, 1998). When Bcl-2 expression is high, Bcl-X_L expression is relatively low and vice versa. The exception is following activation of either T or B cells when Bcl-X_L expression is markedly increased whereas Bcl-2 levels remain relatively unchanged (Boise et al., 1993).

A single study has recently appeared describing the effects of Bid ablation (Yin et al., 1999). Bid knockout mice appear to be resistant to hepatocellular apoptosis and hemorrhagic necrosis induced by injection of antibodies directed against the Fas receptor. Approximately half of the Bid-deficient animals had no apparent liver injury and effector caspases -3 and -7 were not activated although the initiator caspase-8 was processed. Mitochondrial dysfunction (discussed in **sections 1.6** and **1.7.2**) was not detected.

Bax-deficient mice appear to be healthy, indicating that Bax is not essential during development (Knudson et al., 1995). Bax$^{-/-}$ mice display defective reproductive capacities in the testes and seminiferous tubules of males and the ovaries of females resulting from proliferative abnormalities (Knudson et al., 1995). In addition, hyperplasia characterizes T and B lymphocyte populations. Thus, Bax appears to be essential for regulation of reproductive cells and cells of adaptive immunity.

1.6 The Role of Mitochondria in Apoptosis

Mitochondria are acknowledged as the power centres of the cell, satisfying most of the cellular ATP demands consequently allowing cells to live. In the last half decade, however, it has become apparent that mitochondria also figure prominently in the initiation and execution of cell death (for review see Green and Kroemer, 1998; Green, 1998; Green and Reed, 1998; Kroemer, 1998; Kroemer et al., 1998; Kroemer and Reed, 2000; Kroemer et al., 1997; Reed, 1997). Two key events appear to be necessary for apoptosis mediated through mitochondria, loss of the inner mitochondrial membrane potential ($\Delta\Psi_m$) and the efflux of apoptogenic proteins from the mitochondrial intermembrane space to the cytosol. These mitochondrial events as well as the mechanistic aspects contributing to and consequent of mitochondrial involvement in apoptosis are discussed in the next sections.

1.6.1 Mitochondria Structure and Function

Mitochondria are organelles of variable size and shape although their fundamental architecture remains constant (**Figure 1.6-1A**). A smooth outer membrane encloses the mitochondrion. This membrane is permeable to molecules up to ~10 kDa, a result of the presence of non-specific pores made up of the protein porin (also referred to as the voltage-dependent anion channel or VDAC).

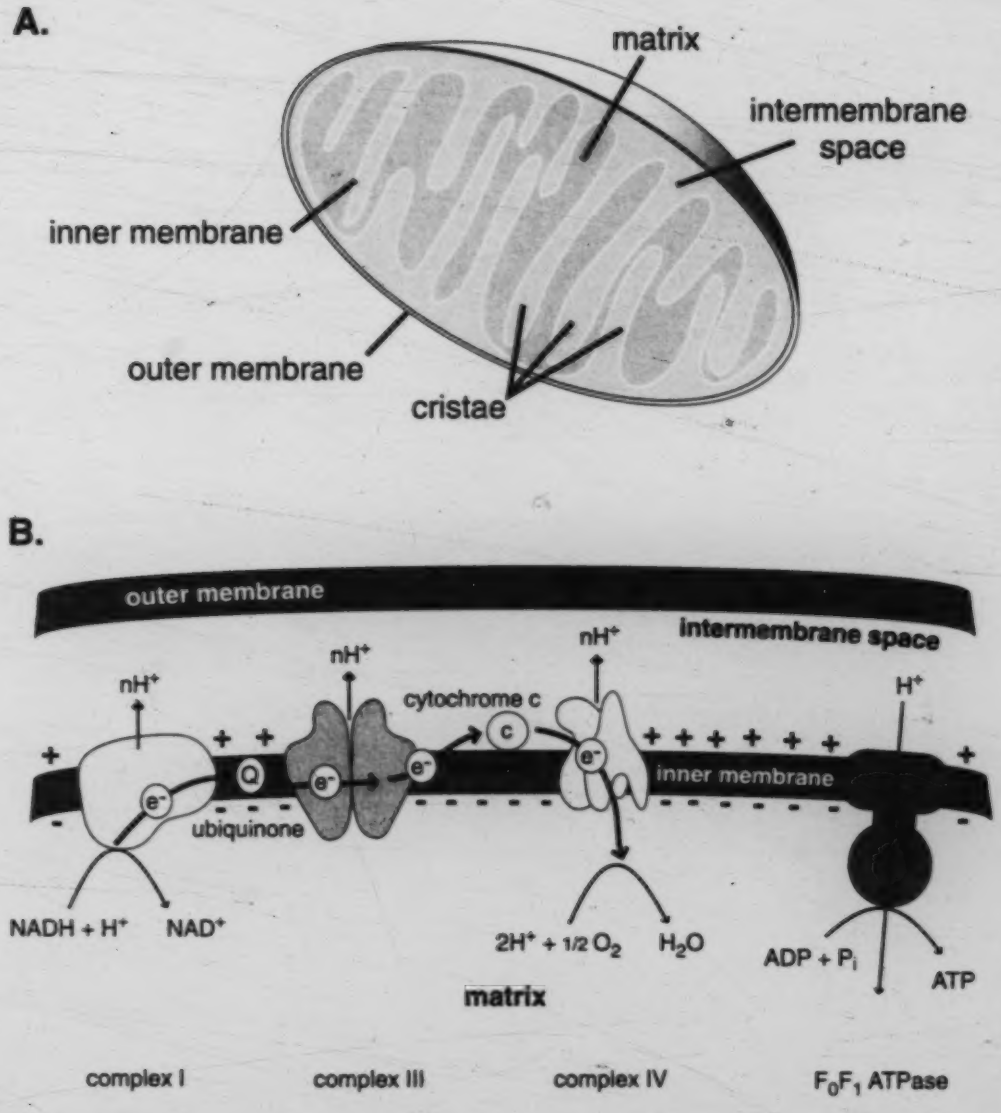


Figure 1.6-1 Mitochondrial Structure and Energy Production

A. Cross section of a mitochondrion showing the different compartments. **B.** Electron transport and the generation of the electrochemical gradient across the inner membrane. Electrons are transferred from NADH to molecular oxygen through a number of redox centres, allowing H^+s to be pumped from the matrix to the to the intermembrane space. Protons are allowed to moved down the electrochemical gradient thus created across this membrane through the F_0F_1ATPase thereby providing energy to generate ATP.

The inner membrane consists of a large number of invaginations, called cristae, which significantly increase the surface area of the inner membrane. This membrane contains most of the proteins that participate in electron transport and oxidative phosphorylation. The inner membrane is essentially impermeable allowing passage only of O_2, CO_2, and H_2O. In addition to respiratory chain proteins, a number of transport proteins are found within the inner membrane including those that regulate the transit of metabolites such as ATP, ADP, pyruvate, Ca^{2+}, H^+, and phosphate.

The inner membrane surrounds the inner compartment of the mitochondrion, called the matrix. The vast majority of matrix proteins are involved in oxidative metabolism, including enzymes of the citric acid cycle and fatty-acid oxidation. Additionally, a number of other molecules are found in the matrix such as the substrates of oxidative metabolism, nucleotide co-factors, inorganic ions and the constituents of the mitochondrial genetic machinery –DNA, RNA and ribosomes.

The impermeability of the inner membrane is central to the respiratory process. During electron transport, ATP synthesis is coupled to the free energy released when electrons from NADH and $FADH_2$ generated during glycolysis and fatty-acid oxidation are transferred to O_2 via a number of protein-bound redox centres (**Figured 1.6-1B**).

During the transfer of electrons to O_2, protons are pumped from the matrix to the intermembrane space through complexes I, III and IV. Consequently, the low $[H^+]$ in the matrix renders it negatively charged relative to the intermembrane space thereby generating a measurable electrochemical H^+ gradient across the inner membrane (the inner mitochondrial membrane potential is represented as $\Delta\Psi_m$). The free energy sequestered by the electrochemical potential is harnessed by a multi-subunit ATPase, the F_oF_1 ATPase, which synthesizes ATP as it transports H^+ back to the matrix. Thus ATP production absolutely requires that the inner mitochondrial membrane be impermeable.

1.6.2 Cytochrome c, the Apoptosome and Cell Death

Cytochrome c is a 15 kDa protein peripherally associated with the inner mitochondrial membrane through ionic interactions that was first identified as a critical component of the electron transport chain (for review see Gonzales and Neupert, 1990) (**Figure 1.6-1**). Apo-cytochrome c is transcribed from a nuclear gene, translated on cytoplasmic ribosomes and exported to mitochondria through a unique mechanism which does not require a signal sequence or the general protein translocation machinery (Mayer et al., 1995). Following transport across the mitochondrial inner membrane, cytochrome c acquires a heme group through the action of a heme lyase (Gonzales and Neupert, 1990). The addition of the heme group alters the protein's structure such that it is unable to escape the intermembrane space. The globular architecture of holo-cytochrome c sequesters the reactive heme group allowing the protein to shuttle electrons between complex III and IV of the electron transport chain. This function of cytochrome c had been recognized for many decades and until recently, cytochrome c research revolved around deciphering the biophysical mechanisms of electron transfer. However, in 1996 a startling new role for cytochrome c in cellular homeostasis emerged, ushering a new wave of cell biological research that has lead to an understanding of what Martin Crompton has recently referred to as the 'Jekyll and Hyde nature of mitochondria' (Crompton, 2000).

While at the Emory University School of Medicine, Xiaodong Wang and co-workers developed a cell-free system for examining apoptosis that utilized extracts from the human cervical carcinoma cell line, HeLa. Using this system, it was discovered that apoptotic activity, as measured by capsase-3 cleavage, DNA fragmentation, and nuclear morphological changes, required mitochondria and the addition of dATP. Fractionation of the extract revealed that three cytosolic components, designated as apoptosis activating factors (Apafs 1-3), were required for apoptotic

activity (Liu et al., 1996). Quite surprisingly, based upon its absorbance spectrum and sequencing information, Apaf-2 was identified as cytochrome c (Liu et al., 1996). It was subsequently determined that cytochrome c release from the intermembrane space to the cytosol was an early and invariant occurrence during apoptosis (Kluck et al., 1997; Kluck et al., 1997; Liu et al., 1996; Yang et al., 1997).

The mechanistic contribution of cytochrome c to the induction of apoptosis became clearer when the identities and functions of the other Apafs were elucidated (**Figure 1.6-2**). Apaf-1 is a homologue of the *C. elegans* Ced-4 protein that regulates programmed cell death in the nematode while Apaf-3 was identified as pro-caspase-9 (Li et al., 1997; Zou et al., 1997). The formation of a protein complex, or apoptosome, consisting of Apaf-1, cytochrome c (Apaf-2) and pro-caspase-9 (Apaf-3) was shown to cause the proteolytic activation of caspase-9 dependent upon the hydrolysis of dATP/ATP by Apaf-1 (Li et al., 1997; Zou et al., 1999). Hydrolysis of dATP/ATP in the presence of cytochrome c leads to Apaf-1 oligomerization and the recruitment of pro-caspase-9, thereby bringing multiple pro-caspase-9 molecules into close proximity, permitting the low intrinsic activity of pro-caspase-9 to effect transactivation (Hu et al., 1999; Pan et al., 1998; Srinivasula et al., 1998; Zou et al., 1999). Subsequently, active caspase-9 dissociates from the complex and can cleave and activate caspase-3, and -7 (Pan et al., 1998; Slee et al., 1999; Srinivasula et al., 1998; Zou et al., 1999). Once activated, caspase-3 may also cleave and activate caspase-2, caspase-6 and caspase-9 (Slee et al., 1999). At least in vitro, caspase-6 can activate the initiator caspases -8 and -10 (Slee et al., 1999). The implication is that caspase-8 and -10 in conjunction with caspase-3, could potentially feedback onto mitochondria thereby establishing feed-forward mechanisms to accelerate cell death.

Microinjection of purified cytochrome c directly into the cytosol of a number of different cell lines has been shown to be sufficient to induce apoptosis

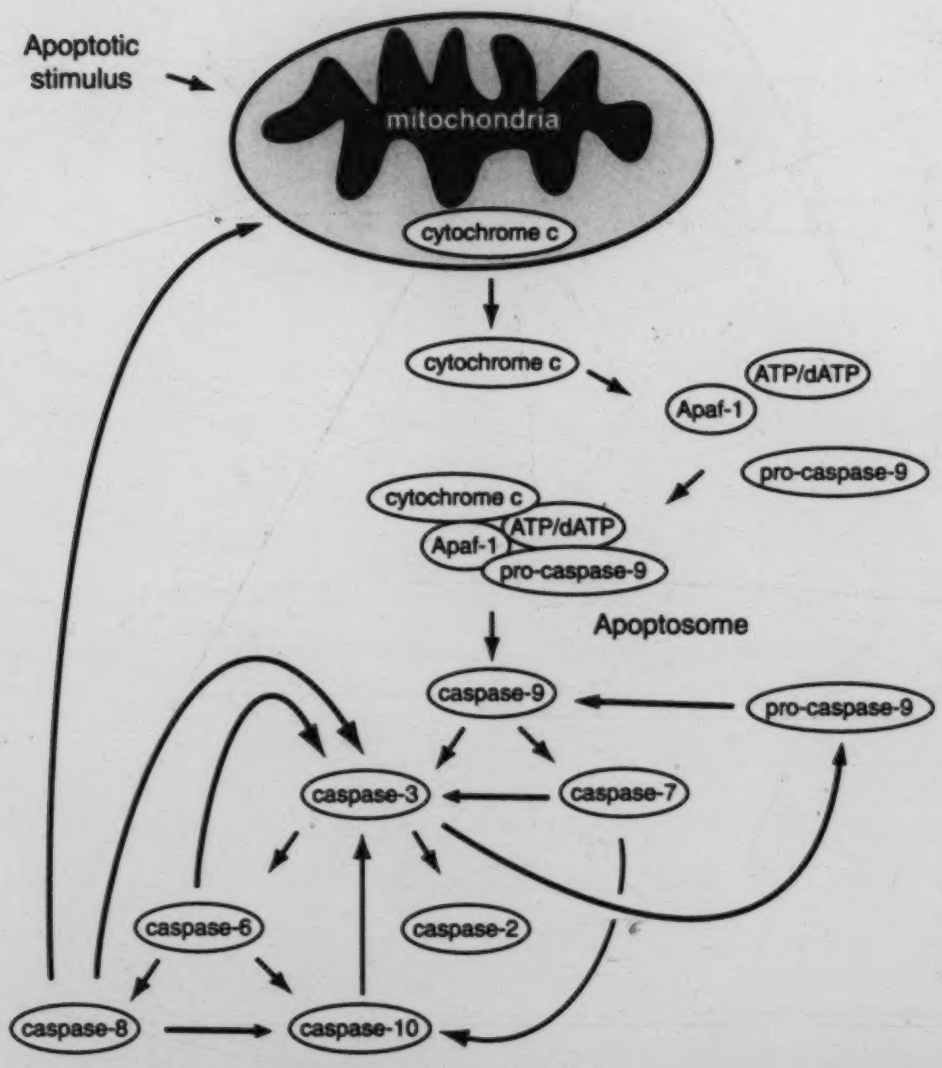


Figure 1.6-2 Cytochrome c Release Leads to Caspase Activation

Following an apoptotic stimulus, cytochrome c is released from the mitochondrial intermembrane space. Once in the cytoplasm, cytochrome c binds to Apaf-1 to initiate a conformational change dependent upon the hydrolysis of dATP/ATP that allows Apaf-1 to oligomerize and bind pro-caspase-9, thereby bringing pro-caspase-9 molecules into close proximity. Pro-caspase-9 transactivates itself, and active caspase-9 is released from the apoptosome to initiate a caspase cascades. Caspase feed-forward loops also exist which likely accelerate the apoptotic process and ensure that the cell is irrevocably committed to death.

(Li et al., 1997; Zhivotovsky et al., 1998). Apoptosis induced in this way was blocked either by Bcl-2 or Bcl-X_L (Li et al., 1997; Zhivotovsky et al., 1998). Recent studies with cell lines derived from cytochrome c knockout mice indicated that the cytochrome c-dependent pathway is essential for apoptosis induced by UV irradiation, serum withdrawal, or staurosporine but not TNFα (Li et al., 2000). In addition, in gene-targeted APAF-1$^{-/-}$ mice, caspase-3 activation does not occur and the mice have severe neurological developmental defects (Cecconi et al., 1998; Yoshida et al., 1998). However, thymocytes and T lymphocytes from these mice showed no impairment of apoptosis induced through the Fas pathway indicating that death receptor-induced apoptosis did not require a mitochondrial pathway in these cells (Cecconi et al., 1998; Yoshida et al., 1998) (Fas-based apoptosis is discussed in **section 1.7.2**).

Data from caspase-9$^{-/-}$ mice parallels that of the Apaf-1 null mice. Embryonic stem cells and embryonic fibroblasts from caspase-9 null mice were found to be resistant to a variety of apoptotic stimuli including UV and gamma irradiation but not to cytotoxic T lymphocytes or TNFα (Hakem et al., 1998). These data support the notion that mitochondrial pathways are important for the execution of apoptosis although they also hint that while cytochrome c release may occur universally, the mitochondrial pathways may not always be required for the ultimate demise of the cell. Since apoptosis is often initiated as a means to eliminate pathogenic organisms, redundancy may have evolved to outsmart the myriad pathogens which themselves have evolved to escape apoptosis induction.

1.6.3 The Permeability Transition

One of the earliest detectable features of apoptosis is the loss of $\Delta\Psi_m$ (for review see Green and Kroemer, 1998; Kroemer et al., 1998; Kroemer and Reed, 2000). $\Delta\Psi_m$ dissipation occurs irrespective of cell type and of the apoptotic stimulus

and can be measured using any of a number of potential-sensitive cationic lipophilic dyes such as rhodamine 123, 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), or 2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI) (Kroemer et al., 1998; Marshall et al., 1992; McCormick, 1990). The lipophilic attributes of these dyes allow them to freely distribute through membranes while their cationic nature ensures that they accumulate in the negatively charged environment of the mitochondrial matrix.

Loss of inner membrane potential has been ascribed to the opening of a large conductance, multi-protein pore spanning the inner and outer membranes called the permeability transition (PT) pore (for review see Crompton, 1999; Zoratti and Szabò, 1995). The core constituents of the pore include matrix cyclophilin D, the adenine nucleotide translocator (ANT) in the inner membrane, and VDAC in the outer membrane. The pore is thought to arise from the association of ANT with VDAC at sites of close proximity between the inner and outer membranes (Crompton, 1999). In isolated mitochondria, PT pore opening is associated with matrix swelling resulting from H^+ gradient dissipation, osmotic dysregulation and solute influx to the matrix (Crompton, 1999; Reed, 1997). Swelling as a secondary consequence of PT pore opening has been offered as one theory for how mitochondria initiate cell death (Vander Heiden et al., 1997). Since the surface area of the inner membrane far exceeds that of the outer membrane, swelling leads to rupture of the outer membrane and the release of proteins from the intermembrane space. Such a mechanism is consistent with studies showing that dozens of proteins, some as large as 80 kDa, are released during mitochondria-induced cell death (Kohler et al., 1999; Patterson et al., 2000; Single et al., 1998). In addition to cytochrome c, the efflux of a number of other apoptogenic proteins including apoptosis inducing factor (AIF) and caspases has also been observed (Ellerby et al., 1997; Kantrow and Piantadosi, 1997; Susin et al., 1999;

Susin et al., 1999). Hence, it appears that at least in some systems, PT pore opening correlates with cytochrome c release and apoptosis.

As an example of PT pore-induced cell death in thymocytes and T cells, inducing the loss of $\Delta\Psi_m$ through stimulation of PT pore opening with protoporphyrin IX (PPIX) leads to the onset of apoptosis (Marchetti et al., 1996; Marchetti et al., 1996; Zamzami et al., 1996). $\Delta\Psi_m$ loss and apoptosis induction can be blocked be preventing PT pore opening with known inhibitors of the PT pore, such as cyclosporine A (which binds to cyclophilins), bongkrekic acid (a ligand of the adenine nucleotide transporter) or chloromethyl-X-rosamine (CMXRos; acts on mitochondrial matrix thiols) (Castedo et al., 1996; Marchetti et al., 1996; Marchetti et al., 1997; Marchetti et al., 1996; Zamzami et al., 1996). Interestingly, $\Delta\Psi_m$ loss is also seen in cells lacking mitochondrial DNA indicating that while mitochondria are central to the execution of apoptosis, all of the relevant participating proteins are encoded by the nuclear genome (Marchetti et al., 1996). Collectively, these data imply that PT pore opening is sufficient to induce apoptosis. However, while $\Delta\Psi_m$ loss is a common feature of apoptosis, it does not always result from PT pore opening (Crompton, 1999; Zoratti and Szabò, 1995). In addition, a number of studies have indicated that cytochrome c release occurs either before or in the absence of detectable $\Delta\Psi_m$ loss hinting that the permeability transition is not the cause of cytochrome c efflux (Bossy-Wetzel et al., 1998; Kluck et al., 1997; Yang et al., 1997).

A second mechanism which has been proposed to explain the release of proteins from the intermembrane space involves the formation of a channel located in the outer membrane (Reed, 1997). Such a channel would necessarily be non-specific and large to accommodate the variety of proteins of variable size that have been identified exiting the intermembrane space following an apoptotic stimulus (Ellerby et al., 1997; Kantrow and Piantadosi, 1997; Kohler et al., 1999; Patterson et al., 2000;

Single et al., 1998; Susin et al., 1999; Susin et al., 1999). In addition, since cytochrome c release precedes $\Delta\Psi_m$ loss in a number of systems, the release of proteins from the intermembrane space requires permeabilizing the outer membrane without disrupting the inner membrane. While the presence of large, non-specific channels best fits with the available data, it is currently unclear how such a channel might operate. However, a potential mechanism for such channel formation is outlined in the next section (**section 1.6.4**).

1.6.4 Bcl-2 Proteins Regulate Mitochondrial Homeostasis

Bcl-2 has been shown to block cytochrome c release from mitochondria as well as the loss of AIF and the disruption of $\Delta\Psi_m$ (Kluck et al., 1997; Susin et al., 1996; Yang et al., 1997). In addition, members of the Bcl-2 family, including Bcl-2, Bcl-X_L, Bid, and Bax, have been implicated in channel formation based on their structures and co-localization with proteins such as VDAC. The next sections outline what is currently known about channel formation in the outer mitochondrial membrane and how Bcl-2 family members may influence the regulation of this pore.

1.6.4.1 The Role of Ion Channel Formation in PT Pore Regulation

No structural information is currently available for Bcl-2. Nevertheless, Bcl-2 and Bcl-X_L are thought to be structurally homologous and the solution structure for Bcl-X_L has been solved (Muchmore et al., 1996). Five amphipathic helices surround two central hydrophobic α-helices. BH1, BH2, and BH3 are spatially close creating a hydrophobic binding pocket for BH3 domains of other Bcl-2 family members (Sattler et al., 1997). The BH4 domain consists of a side helix, α1, while a 60 residue loop between α1 and α2 is unstructured and non-essential for anti-apoptotic function (Chang et al., 1997; Muchmore et al., 1996). The overall structure is reminiscent of bacterial toxins such as diptheria toxin and the colicin family of toxins which act through

a mechanism dependent on pore-formation (Muchmore et al., 1996). The structural similarity to pore-forming toxins led to the notion that Bcl-X_L may form ion channels through membrane insertion of its two central helices. Indeed, Bcl-2, Bcl-X_L, Bax, as well as Bid have all been shown to have channel forming abilities in synthetic lipid membranes (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1999; Schendel et al., 1997; Schlesinger et al., 1997). The channels formed by Bcl-2 family proteins have a variety of ion selectivities and pH optima. Consistent with a channel-forming role for Bcl-2 in regulating apoptosis, removal of the α5 and α6 helices of Bcl-2 prevented channel formation in vitro and abrogated its anti-apoptotic function in whole cells (Matsuyama et al., 1998; Schendel et al., 1997). In addition, Bcl-2 blocked Bax channel-forming activity in synthetic lipid membranes (Antonsson et al., 1997).

How channel formation may regulate apoptosis is unclear. However, Bcl-2, Bcl-X_L and Bax have all been shown to co-localize with proteins comprising the PT pore (Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999) Recently, Bcl-X_L was shown to regulate closing of VDAC whereas Bax and another pro-apoptotic Bcl-2 family member, Bak, stimulated opening of VDAC and allowed passage of cytochrome c from liposomes (Shimizu et al., 1999). Normally, VDAC is no larger than 1.5 nm in the open conformation. To allow passage of cytochrome c in its heme-containing holo form, a pore of at least 3 nm or larger diameter would be required. Bax and Bak appear to increase the pore diameter of VDAC, allowing cytochrome c to escape. Bax has been shown to cause cytochrome c release and apoptosis when over-expressed in yeast (Priault et al., 1999). VDAC-deficient yeast mutants were unable to release cytochrome c when human Bax was added illustrating the requirement for VDAC in Bax-mediated cytochrome c release (Shimizu et al., 1999). Recently, the BH4 domain of Bcl-2 or Bcl-X_L was shown to close VDAC, thereby blocking cytochrome c release and $\Delta\Psi_m$ loss (Shimizu et al., 2000). Therefore, channel formation and/or regulation by

Bcl-2 family members appears to influence the release of mitochondrial proteins including cytochrome c although the mechanistic details underlying cytochrome c release remain hazy.

1.6.4.2 The Role of Heterodimerization of Bcl-2 Family Members

An additional mechanism by which Bcl-2 proteins have been hypothesized to regulate mitochondrial function is through protein-protein interactions. The importance of Bcl-2 family dimerization has already been alluded to in **section 1.5.2** where it was introduced that Bid and Bax were first identified by their abilities to heterodimerize with Bcl-2. Dimerization involves the conserved BH domains. The BH1, BH2 and BH3 domains of anti-apoptotic Bcl-2 proteins including Bcl-2 and Bcl-X_L are thought to form a hydrophobic binding pocket for the BH3 domains of pro-apoptotic Bcl-2 family members such as Bid, Bax and Bak (Chittenden et al., 1995; Sattler et al., 1997; Simonen et al., 1997; Zha et al., 1996). Such binding allows for heterodimerization between pro- and anti-apoptotic family members and may contribute to the maintenance of cellular homeostasis. Indeed, heterodimerization has been proposed to govern a rheostat model where the levels of pro- and anti-apoptotic Bcl-2 family members ultimately determine cell fate (Kroemer, 1997; Oltvai et al., 1993). Such a rheostat model would be consistent with VDAC regulation.

Support for the role of heterodimerization in regulating apoptosis comes from studies where mutations in the BH3 domain of Bax or Bak prevented heterodimerization and additionally, blocked the apoptotic activity these proteins (Sattler et al., 1997; Simonen et al., 1997). In addition, mutations in Bid's BH3 domain which prevented its binding to Bcl-2/Bcl-X_L also blocked cytochrome c release and apoptosis (Gross et al., 1999; Luo et al., 1998; Wang et al., 1996). However, the exact role of heterodimerization between Bcl-2 family members remains unclear since Bax-

mediated apoptosis has also been shown to occur independently of heterodimerization (Minn et al., 1999).

The overall implication of the rheostat model is that cell survival is promoted under conditions where the levels of anti-apoptotic Bcl-2 family members prevail while cell death occurs when the balance of proteins favours pro-apoptotic family members. For example, when Bax is preferentially expressed, Bax homodimers form and death is favoured. In contrast, when Bcl-2 is preferentially expressed, Bcl-2 homodimers tip the balance in favour of cell survival. Pro-apoptotic BH3 only proteins such as Bid do not homodimerize but do heterodimerize with Bcl-2 thereby inhibiting their death promoting activities (Kelekar et al,, 1997; Sattler et al., 1997). Alternatively, BH3-only containing proteins may bind to other pro-apoptotic Bcl-2 family members to stimulate or potentiate their activities (Kelekar and Thompson, 1998).

In contrast with the BH1, BH2 and BH3 domains, the BH4 domain is only conserved between anti-apoptotic Bcl-2 family members (**Figure 1.5-3**), the implication being that this domain is important for the anti-apoptotic activity of these proteins. Support for such a role of the BH4 domain comes from studies where its deletion from Bcl-2 or Bcl-X_L abrogated the anti-apoptotic activity of these proteins (Borner et al., 1994; Hanada et al., 1995; Huang et al., 1998; Hunter et al., 1996; Lee et al., 1996). The BH4 domain may be important for regulating cell responses to the extracellular environment mediated through a number of signaling molecules that may influence life/death decisions. For example, the BH4 domain has been reported to bind to calcineurin (Shibasaki et al., 1997) and Raf-1 (Wang et al., 1996) to affect cell signaling pathways. Furthermore, the BH4 domain of Bcl-X_L or Bcl-2 has been shown to interact with Ced-4 (Huang et al., 1998)/Apaf-1 (Hu et al., 1998; Newmeyer et al., 2000; Pan et al., 1998) and this interaction is thought to block apoptosis by sequestering Apaf-1 away from apoptosome formation even when cytochrome c is lost from mitochondria.

However, other studies suggest that Bcl-2 and Bcl-X_L do not directly interact with Apaf-1 (Hausmann et al., 2000; Moriishi et al., 1999) while another reported that while Bcl-X_L and Apaf-1 did interact, Bcl-X_L was unable to prevent caspase activation mediated by the addition of cytochrome c in a cell-free system (Newmeyer et al., 2000).

1.7 Current Models of CTL-Mediated Cell Death

The contemporary view of CTL-mediated killing is that there are two primary mechanisms utilized by CTL to eliminate targets. These are the granule-mediated and Fas-dependent pathways. The next sections recapitulate what is currently known about each of these pathways.

1.7.1 Fas-Mediated Cytotoxicity

Fas(CD95/APO-1)-mediated cytotoxicity is invoked largely during thymocyte maturation and to down-regulate the immune response following activation (Nagata and Golstein, 1995; Peter and Krammer, 1998). CTL primarily utilize the granule-dependent pathway to kill targets, however, arguably one of the best-understood pathways of apoptosis is killing mediated through the Fas receptor. Many of the proteins involved in Fas-mediated killing have been identified and the molecular ordering of these proteins and their roles in apoptosis have been well characterized. As we shall see in later chapters, there is overlap of many of the proteins recruited by both the granule-dependent and Fas pathways. For these reasons, a brief introduction to the Fas pathway is advantageous. The next sections outline what is currently known about the molecular events of Fas-mediated killing.

1.7.1.1 Fas, Fas Ligand and the Molecular Events of Fas-Mediated Killing

Granule-mediated killing is dependent upon extracellular Ca^{2+} both for the process of degranulation (**section 1.2.3**) and for the conformational change allowing perforin to insert into the target cell membrane (**section 1.3.1.2**). However, it had been

recognized for some time that certain CTL were able to kill in the absence of Ca^{2+}, perforin or granzymes (Ostergaard et al., 1987, Allbritton et al., 1988; Berke and Rosen, 1988; Berke et al., 1993; Helgason et al., 1992). This Ca^{2+}-independent killing is now known to occur through interactions between Fas ligand (FasL) expressed on the CTL and the Fas receptor expressed on the target cell surface (for review see Nagata and Golstein, 1995; Peter and Krammer, 1998). FasL is a ~40 kDa type II transmembrane protein of the TNF family that can also be expressed and secreted in soluble form (Nagata and Golstein, 1995). Fas, on the other hand, is a ~32 kDa type I transmembrane protein of the TNF receptor family that is known to exist only in a membrane-bound form.

The binding of the Fas receptor either by FasL or by anti-Fas IgM antibodies leads to receptor oligomerization (**Figure 1.7-1**) (Peter and Krammer, 1998). Oligomerization, in turn, allows the adapter protein, Fas-associated death domain (FADD)MORT1 to bind to the cytoplasmic domain of Fas through homologous death domains found in both FADD and Fas (Boldin et al., 1996; Boldin et al., 1995; Chinnaiyan et al., 1995; Kischkel et al., 1995). This protein scaffolding is referred to as the death-inducing signaling complex (DISC) (Kischkel et al., 1995). FADD contains death effector domains (DEDs) which foster recruitment to the DISC of initiator pro-caspase-8 molecules that contain homologous DEDs within their pro-domains (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996; Scaffidi et al., 1997). This allows many pro-caspase-8 molecules to come into closer proximity. Pro-caspase-8 has an intrinsic, but low, level of activity in the absence of activation that allows for trans-activation when many pro-molecules are brought near to one another (Muzio et al., 1998). In cell types where pro-caspase-8 recruitment is efficient (designated type I cells), active caspase-8 directly cleaves and activates caspase-3 to initiate many of the downstream morphologies of apoptosis (Scaffidi et al., 1998). By contrast, in cells

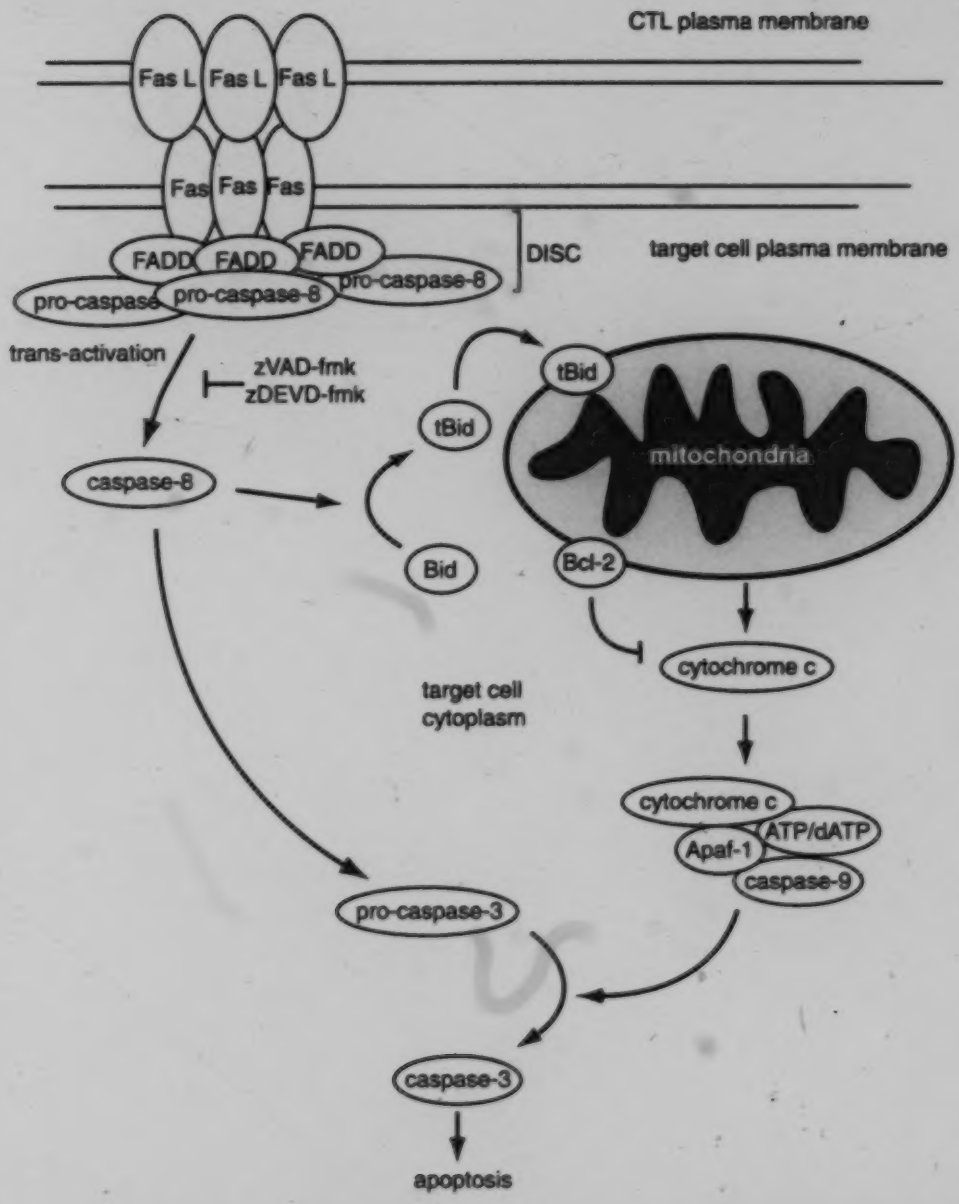


Figure 1.7-1 The Molecular Basis of Fas-Mediated Killing

The molecular events leading to caspase-3 activation that occur during Fas-mediated killing in both type I and type II cells are illustrated. In type I cells, activation of caspase-8 leads to direct activation of caspase-3. In type II cells, caspase-3 activation requires a mitochondrial pathway which can be antagonized by Bcl-2. See **section 1.7.1** for more details.

in which pro-caspase-8 recruitment to the DISC is inefficient (type II cells), caspase-3 activation occurs through a mitochondrial pathway (Scaffidi et al., 1998). In this pathway, caspase-8 cleaves the pro-apoptotic Bcl-2 family member Bid in the cytosol to generate a 15 kDa cytosolic truncated fragment (tBid) which translocates to mitochondria (Li et al., 1998; Luo et al., 1998). Once there, tBid induces the efflux of cytochrome c through an as yet undefined mechanism. Released cytochrome c acts a co-factor within an apoptosome comprised of Apaf-1, pro-caspase-9 and ATP/dATP to cause activation of caspase-3 (see **section 1.6.2**) (Hu et al., 1999; Li et al., 1997; Saleh et al., 1999; Zou et al., 1997; Zou et al., 1999).

The fact that there are different cell-types with different molecular requirements for killing mediated through the Fas pathway largely explains the differential sensitivity of the Fas pathway to Bcl-2 over-expression. Some researchers have reported that Bcl-2 does not inhibit Fas-mediated killing in some cells (now recognized as type I cells) while others have reported that the Fas pathway is blocked by Bcl-2 in other cells (now recognized as type II cells). In type II cells, where mitochondria play a prominent role in transducing the death signal, Bcl-2 blocks all measures of apoptosis whereas in type I cells, mitochondrial events are blocked but DISC formation occurs and pro-caspase-8 is still processed as is caspase-3 (Scaffidi et al., 1998).

1.7.2 Granule-Mediated Cytotoxicity

While CTL may employ the Fas pathway to kill targets, in a physiological setting Fas-mediated killing is largely reserved for the removal of thymocytes during T cell maturation and for the elimination of activated T cells following an adaptive immune response (Nagata and Golstein, 1995). The primary mechanism CTL adopt for the elimination of targets involves granule-mediated cytotoxicity. The molecular basis of granule-mediated killing as it was understood prior to the collection of data presented in this thesis is outlined in the following sections.

1.7.2.1 Granule Exocytosis and the Lethal Hit

As has been described (**section 1.3**), the lytic granules of CTL contain granzymes A, B and perforin in particular abundance following activation. The "lethal hit" model of granule-mediated cytotoxicity, first proposed by Henkart (Henkart, 1985), stated that following granule exocytosis, perforin formed channels in the target cell membrane through a Ca^{2+}-dependent mechanism (Atkinson and Bleackley, 1995; Darmon et al., 1999) (**Figure1.7-2**). It was through these poly-perforin channels that the granzymes gained entry to the target cell cytoplasm and substrates. Target cell substrates included caspases, and in particular caspase-3, that, once processed by granzymes went on to cleave cellular targets to mediate the known morphologies of target cell death.

We now recognize that the entry of granzymes into the target likely involves routes other than poly-perforin channels. Receptor-mediated endocytosis has been proposed as a mechanism for granzymes to enter targets and there is growing evidence to support such a route for granzymes (Edwards et al., 1999; Pinkoski et al., 1998). Indeed the mannose-6-phosphate receptor has recently been identified as a target cell surface receptor to which granzyme B binds, allowing this protein entry into the cell in the absence of perforin (Motyka et al., 2000). As outlined in **section 1.3.1.2**, the role of perforin in granule-dependent killing is not well defined, however, it is clear that perforin is absolutely required. While likely not necessary for the entry of granzyme B at the level of the plasma membrane, one clear function of perforin is to release granzyme B from intracellular cytoplasmic vesicles into which granzyme B accumulates following receptor binding and internalization (Pinkoski et al., 1998). Although it has never been shown directly, once released from these vesicles, granzyme B is presumed to enter the cytoplasm based on the fact that a number of cytoplasmic substrates that are cleaved in vitro by granzyme B are also processed in targets.

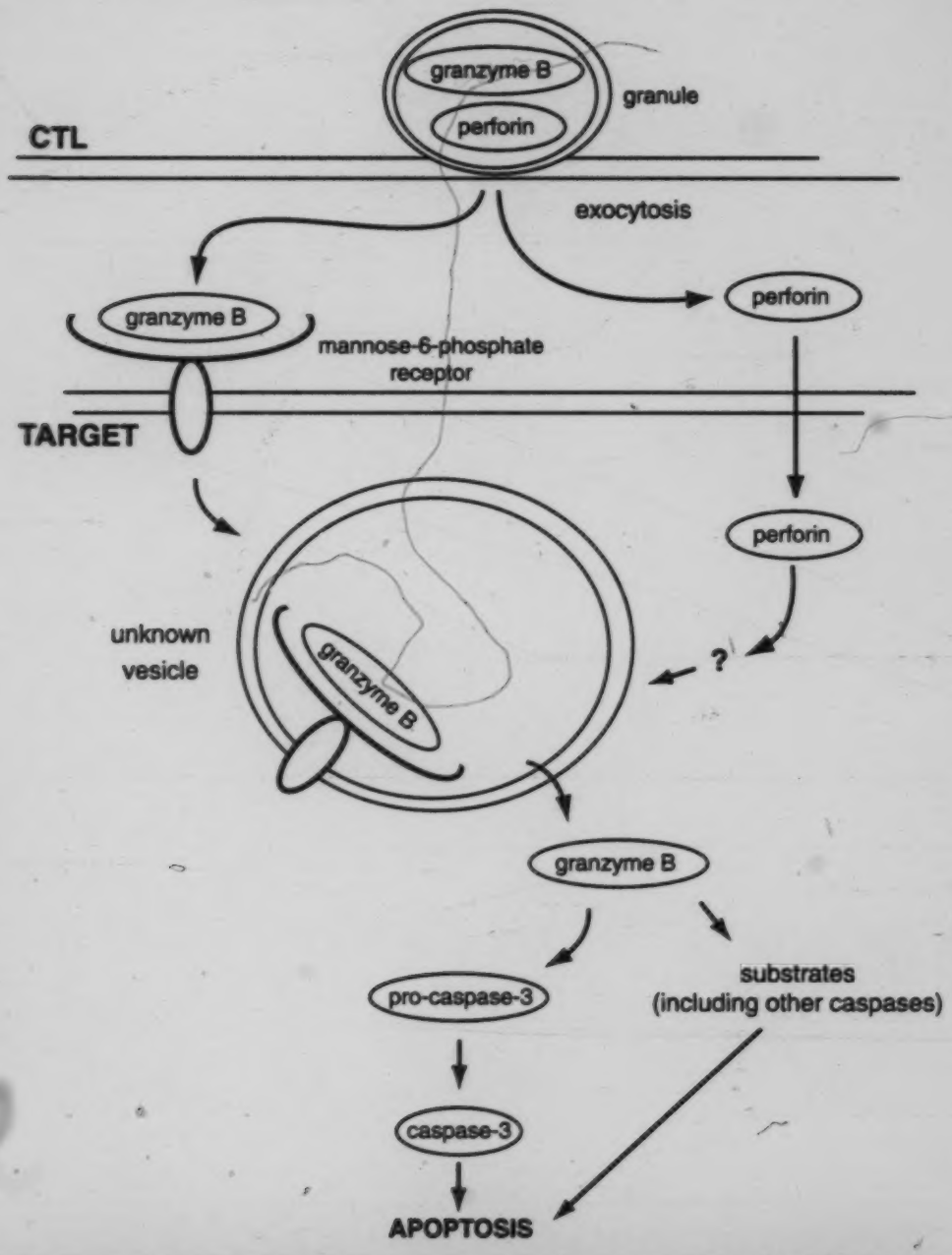


Figure 1.7-2 The Molecular Basis of Granule-Mediated Killing

Granzyme B and perforin are released during granule exocytosis. Granzyme B binds to the mannose-6-phosphate receptor and remains sequestered in an unknown intracellular vesicle until released to the cytoplasm by the effects of perforin. Once released, granzyme B cleaves cellular substrates, including caspase-3, to induce target cell apoptosis.

These substrates include caspases-1, -3, -6, -7, -8 (Atkinson et al., 1998; Barry et al., 2000; Chinnaiyan et al., 1996; Darmon et al., 1996; Shi et al., 1996) as well as gelsolin (Pinkoski and Bleackley, unpublished observations). Moreover, it has been shown that granzyme B directly activates caspase-3 in targets, further supporting the requirement for its release from vesicles to the cytoplasm and additionally, directly linking granzyme B activity with caspase activation at least in some cell types (Atkinson et al., 1998). However, as we shall see, data presented in this thesis indicates that while granzyme B can directly process caspase-3, a number of granzyme-initiated mitochondrial events may be required before the enzymatic activity of this processed caspase is released.

1.7.2.2 The Role of Bcl-2 and Caspases in Regulation of Granule-Mediated Killing

As with its effects on Fas-based killing, Bcl-2 appears to have differential capacities to regulate granule-mediated cell death. In some systems, Bcl-2 appears to offer partial protection or no protection at all against DNA fragmentation and cell lysis (Sutton et al., 1997). In contrast, in other cell-types Bcl-2 blocks early DNA fragmentation although with longer incubations this inhibition can be overcome (Renvoizé et al., 1997). Despite impairing DNA fragmentation, Bcl-2 appears to provide no protection against membrane damage although this membrane damage has been largely attributed to the membraneolytic effects of perforin (Renvoizé et al., 1997; Torigoe et al., 1994). In other systems, Bcl-2 protects targets from both DNA fragmentation and membrane damage (Chiu et al., 1995).

It is now recognized that stimulation of the Fas receptor can impinge upon distinct pathways in different cell-types (i.e. type II cells recruit a mitochondrial pathway whereas type I cells do not). It appears that this variance largely explains the differential ability of Bcl-2 to regulate Fas-mediated killing since type I cells that do not require signaling through mitochondria are largely impervious to the effects of Bcl-2 over-expression. Hence, the differences seen in the ability of Bcl-2 to regulate granule-

dependent killing may also be explained through the involvement of mitochondrial pathways. It is known that granzyme B can directly activate caspase-3, however, it may also be possible that other granzyme B-dependent pathways exist within targets that employ signaling through mitochondria.

In addition to the discrepancies Bcl-2 regulation appears to create, recent studies have appeared that question the absolute requirement for caspases during CTL-mediated death (Sarin et al., 1998; Sarin et al., 1997). In these studies, DNA fragmentation was blocked by pharmacological and viral caspase inhibitors but targets ultimately died through lytic processes reminiscent of necrosis. These studies suggest that the activation of caspases by granzymes ultimately determine whether a cell dies by apoptosis or through necrosis. One simple method to induce necrosis would be to disrupt energy production by mitochondria either through gross mitochondrial membrane damage or through subtler processes such as cytochrome c release and the subsequent disruption of electron transport. However, prior to the acquisition of data presented in this thesis, the explanations for how Bcl-2 or the caspases might influence granule-dependent killing were unknown.

1.8 Thesis Objectives

When I began the studies reported in this thesis, mitochondria were just coming to the forefront of apoptosis research. Initially, the loss of $\Delta\Psi_m$ was recognized as an early and invariant event in response to a variety of apoptotic stimuli. Shortly thereafter, the release of apoptogenic proteins from mitochondria, and in particular cytochrome c, was also observed. Although the mechanistic details underlying these events and the relative contributions of each to the initiation of apoptosis are still controversial, it is now clear that mitochondria are central to the apoptotic response.

Preliminary data in our lab suggested that the involvement of mitochondria might explain irregularities in the model of CTL-mediated killing as it existed at that time. If granzyme B was able to act directly on caspase-3 to elicit activation of this protein, why was Bcl-2 over-expression able to block granzyme-dependent killing in some cell types and why was caspase-3 activation prevented under these circumstances? The available data indicated only that granzyme B could directly activate caspase-3. Beyond this information, whether Bcl-2 might interfere with this processing step was hotly debated and the possible mechanisms that could explain Bcl-2 inhibition when it occurred were ethereal. The known localization of Bcl-2 to mitochondria coupled with the newly emerging prominence of mitochondria in cell death suggested that these organelles could be a focal point for regulating CTL-mediated target cell death.

The specific objectives of this thesis as they relate to mitochondrial involvement of CTL-mediated killing follow. It is important to recognize that the objectives, as laid out here, were far from solidified during the early stages of my work. The tremendous quantity of new information relating to apoptosis that appeared in the literature over the last four years, as well as my own acquisition of data guided the latter stages of research.

Objectives:

i) To establish a link between granzyme B and mitochondrial pathways of target cell death.

ii) To determine if Bcl-2 influences granzyme B-dependent killing and if so, at what stage of the cell death program was Bcl-2 involved and how.

iii) To elucidate the mechanistic aspects of mitochondrial involvement in granzyme B-mediated killing.

Chapter 2
Materials and Methods[1]

[1] Portions of this chapter have been previously published: Jeffrey A. Heibein, Michele Barry, Bruce Motyka and R. Chris Bleackley (1999) Granzyme B-Induced Loss of Mitochondrial Inner Membrane Potential ($\Delta\Psi_m$) and Cytochrome c Release Are Caspase Independent, *Journal of Immunology*, **163**: 4683-4693. *Copyright 1999. The American Association of Immunologists*; Jeffrey A. Heibein, Ing Swie Goping, Michele Barry, Michael J. Pinkoski, Gordon C. Shore, Douglas R. Green, and R. Chris Bleackley (2000) Granzyme B-Mediated Cytochrome c Release is Regulated by the Bcl-2 Family Members Bid and Bax. *J. Exp. Med.*, **192**, 1391-1401. *Copyright 2000. The Rockefeller University Press.*

Chapter 2: Materials and Methods

2.1 Cell Lines

2.1.1 Target Cell Lines

2.1.1.1 Jurkat Cells

The human T cell lymphoma line, Jurkat (American Type Culture Collection (ATCC), Manassas, VA), was grown in RPMI-1640 (Gibco BRL, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT) and 100 μM 2-mercaptoethanol. This growth medium is referred to as RHFM.

2.1.1.2 HeLa Cells

The human cervical carcinoma line, HeLa (ATCC), was grown in RPMI-1640 supplemented with 10% (v/v) FBS. This growth medium is referred to as RHF.

2.1.1.3 Bcl-2 Stable Transfectants

The Bcl-2 gene was subcloned into the Xho I site of the eukaryotic expression vector BMGneo containing a neomycin resistance gene (Karasuyama and Melchers, 1988). Jurkat cells were stably transfected with 10 μg of Not I linearized DNA by electroporation (250 V, 250 μF). Stably transfected cells were selected in RHFM containing 1 mg/ml of G418 (geneticin; Gibco BRL) and cloned by limiting dilution. Following selection, cells were routinely grown in RHFM containing 0.8 mg/ml of G418.

2.1.1.4 GFP Stable Transfectants

Jurkat cells were electroporated (250 V, 250 μF, time constant ~10) either with 10 μg of QIAGEN-purified pEGFP-N1 plasmid (CLONTECH, Palo Alto, CA) or with 10 μg of pEGFP-N1 plus 10 μg of the Bcl-2 plasmid outlined in **section 2.1.1.2**. Both transfected plasmids contained a neomycin-resistance gene. Following selection in

G418 (1 mg/ml), individual clones transfected with pEGFP-only (JpEGFP), clones transfected with pEGFP and the BMGneo empty vector (JpEGFPneo), or clones transfected with both JpEGFP and Bcl-2 (JpEGFPBcl-2) were analyzed for green fluorescent protein (GFP) expression using flow cytometry. Clones were chosen based upon similar levels of GFP expression (where mean fluorescence intensity of all clones chosen was approximately 10^2-10^3 logarithmic units). This provided us with a number of clones possessing an intermediate green phenotype. In addition, these clones were not excessively green thereby preventing the green GFP emission wavelengths from bleeding into other channels when utilizing the cells as targets for measuring other parameters with additional fluorescent dyes. The JpEGFPBcl-2 clones were further screened for Bcl-2 expression by western blotting using an anti-Bcl-2 antibody (2 µg/ml). Bcl-2 clones were chosen based upon their enhanced expression relative to wild-type Jurkats, the GFP-expressing Jurkats (JpEGFP) and the vector-only JpEGFPneo clones (data not shown). JpEGFP clones were maintained in RHFM supplemented with 0.8 mg/ml geneticin (Gibco BRL).

2.1.2 Effectors

Human cytotoxic T lymphocytes (hCTL) were isolated from peripheral blood by centrifugation through Ficoll (Pharmacia Biotech Inc., Baie d'Urfé, QC). Lymphocytes were prepared according to (Atkinson et al., 1998). Briefly, lymphocytes were incubated at $4x10^5$ cells/ml with irradiated, Epstein-Barr virus-transformed RPMI-8666 cells (5000 rad) at a 5:1 ratio (lymphocytes:RPMI-8666). Following three days in co-culture, CTL were purified from RPMI-8666 cells by centrifugation through Ficoll and subsequently maintained in RPMI-1640 containing 10% (v/v) fetal bovine serum and 90 U/ml of IL-2 (Chiron Corporation, Emeryville, CA, USA). All cell lines were maintained at 2.5-$5x10^5$ cells/ml in a humidified atmosphere of 5% CO_2.

2.2 Reagents

2.2.1 Antibodies

2.2.1.1 Primary Antibodies

The monoclonal anti-human Fas IgM antibody (clone CH11) was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The monoclonal anti-human cytochrome c antibody recognizing denatured cytochrome c (clone 7H8.2C12) was from Pharmingen (Mississauga, ON, Canada). Rabbit polyclonal anti-Bax antiserum (N-20) was from Santa Cruz (Santa Cruz, CA, USA). The affinity purified polyclonal rabbit anti-caspase-3 antibody (recognizing the p17 cleavage product of caspase-3) was provided by Don Nicholson (Merck Frosst Therapeutic Centre for Research, Pt. Claire, PQ, Canada). Xiaodong Wang (Howard Hughes Medical Institute, Dallas, TX, USA) kindly provided the rabbit anti-Bid antiserum. The mouse monoclonal anti-Bcl-2 antibody (clone 124) was from Upstate Biotechnology.

2.2.1.2 Secondary Antibodies

The goat anti-mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from BioRad Laboratories (Canada) Ltd. (Mississauga, Ontario, Canada).

2.2.2 Expression and Purification of Recombinant Bid

Xiaodong Wang (Howard Hughes Medical Institute, Dallas, TX, USA) provided Bid (rBid) in the pET-15b vector his-tagged human recombinant. It was expressed in competent BL21 E. coli and purified as described (Luo et al., 1998).

2.2.3 Other Reagents

Human granzyme B was purified from human YT-Indy cells according to Caputo et al., (Caputo et al., 1999). Chris Froelich at Northwestern University supplied

purified human perforin and replication-deficient adenovirus type 2. Replication-deficient adenovirus (type 5) was a generous gift from Jack Gauldie, (McMaster University, Hamilton, ON). Kamiya Biomedical, Seattle, WA, USA, supplied the benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) and benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (zDEVD-fmk). Sigma Chemical Corporation (Oakville ON, Canada) provided all other reagents unless otherwise stated.

2.3 Induction and Measurement of Apoptosis In Vitro

2.3.1 Addition to Targets of Granzyme B/Perforin/Adenovirus

Jurkat cells were washed three times in serum-free RPMI-1640 and resuspended at $2x10^6$ cells/ml in serum-free RPMI-1640 containing 1% BSA (w/v). To 500 µl of cells at $2x10^6$ cells/ml was added 250 µl of high-Ca^{2+} Hepes (20 mM Hepes pH 7.4, 150 mM NaCl, 1% BSA (w/v) and 5 mM $CaCl_2$). Samples treated with granzyme B and/or adenovirus received a further 250 µl of Ca^{2+}-free Hepes (20 mM Hepes, pH 7.4, and 150 mM NaCl and 1% BSA (w/v)) bringing the total assay volume to 1 ml with a final cell concentration of $1x10^6$ cells/ml. Granzyme B and adenovirus were added directly to final concentrations of 1µg/ml and a multiplicity of infection (MOI) of 10 plaque forming units(pfu)/cell respectively. Samples treated with perforin received 250 µl of Ca^{2+}-free Hepes containing sublytic doses of perforin (100-200 hemolytic units (HU) (Froelich et al., 1996); sublytic refers to an amount of perforin giving less than 10% non-specific lysis). Cells were incubated at 37°C for 2 hours unless otherwise indicated. Following this incubation, 200 µl aliquots containing ~$2x10^5$ cells each were removed to assess DNA fragmentation, phosphatidylserine externalization, ROS production and mitochondrial $\Delta\Psi_m$ loss as outlined below.

2.3.2 Analysis of DNA fragmentation and Phosphatidylserine Externalization

DNA fragmentation was determined using terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end labeling (TUNEL) (Gavrieli et al., 1992). TUNEL materials were supplied by Boehringer Mannheim and used as per the manufacturer's instructions. Phosphatidylserine externalization (Fadok et al., 1992; Koopman et al., 1994) was measured using either the ApoAlert™ Annexin V Apoptosis Kit (CLONTECH, Palo Alto, CA) or FITC-annexin V (Pharmingen, Mississauga, ON) as per the manufacturers' instructions. Both TUNEL and annexin V binding were quantified using flow cytometric analysis by examining 10,000 events on a Becton Dickinson FACScan with an excitation wavelength of 488 nm. The emission wavelengths for both the dUTP-FITC and FITC-annexin V were detected through the FL1 channel equipped with a 530 nm (20 nm band pass) filter. Data was acquired and analyzed with CELLQuest software (Beckton Dickinson, Mississauga, ON). Cells deemed TUNEL or FITC-annexin V positive were those displaying fluorescence greater than the fluorescence of controls in the absence of apoptotic induction (control).

2.3.3 Analysis of $\Delta\Psi_m$ Loss and ROS Production

The mitochondrial inner transmembrane potential ($\Delta\Psi_m$) was monitored using 40 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Molecular Probes, Eugene, OR) and reactive oxygen species (ROS) production was determined using 2 μM hydroethidine (HE) (Molecular Probes, Eugene, OR). $DiOC_6(3)$, HE and the positive control for $\Delta\Psi_m$ loss, carbonyl cyanide m-chlorophenyl hydrazone (mClCCP, 5 μM), were added 15 minutes prior to the 2 hour end-point of the apoptosis assay and cells were then maintained for a further 15 minutes at 37°C followed by flow cytometric analysis. $DiOC_6(3)$ was detected through the FL1 channel and HE was detected through the FL2 channel equipped with a 585 nm (20 nm band pass) filter. $DiOC_6(3)^{low}$

cells were those displaying $DiOC_6(3)$ fluorescence less than the fluorescence of control cells in the absence of the apoptotic stimulus. HE^+ cells were those displaying HE fluorescence greater than control cells in the absence of apoptotic induction.

2.3.4 Analysis of Cytochrome c Release in Whole Cells

Jurkat cells ($1x10^6$ cells/ml in serum-free RPMI-1640; 4 ml/sample) were treated with granzyme B (1 µg/ml) and adenovirus (MOI=10 pfu/cell) for the times indicated. Anti-Fas-treated cells (also 4 ml of cells at $1x10^6$ cells/ml in serum-free RPMI-1640) received 500 ng/ml of anti-human Fas IgM for the indicated times. Following treatment, cells were washed twice with PBS and resuspended in 200 µl of digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 190 µg/ml digitonin). Digitonin is a weak nonionic detergent which, at low concentrations, selectively renders the plasma membrane permeable, releasing cytosolic components from cells but leaving other organelles intact (Adam et al., 1990). After 5 minutes on ice, cells were spun for 5 minutes at 14,000 rpm at 4°C in an Eppendorf microcentrifuge. Supernatants were transferred to fresh tubes and the pellets resuspended in Triton lysis buffer (25 mM Tris-HCl, pH 8.0, 0.1% (v/v) Triton X-100). Aliquots (70 µl) of both pellet and supernatant for each sample were added to 30 µl of sodium dodecylsulphate (SDS)-loading buffer (0.5 M Tris-HCl, pH 6.8, 1 M 2-mercaptoethanol, 10% (w/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and boiled for 10 minutes. Boiled samples (25 µl for pellets and 50 µl for supernatants) were loaded onto 15% polyacrylamide gels followed by electrophoresis and transfer to nitrocellulose membranes (Micron Separations Inc., Westborough, MA). Membranes were blocked overnight at 4°C in PBS containing 0.1 % (v/v) Tween-20 (Fisher Scientific, Nepean, ON, Canada) and 5 % (w/v) milk proteins (Carnation, Don Mills, ON, Canada). Blocked membranes were incubated with a monoclonal anti-human cytochrome c antibody (1:2000 dilution in PBST) for 1 hour at room temperature. Membranes were washed 3

times (5 minutes each) with PBST followed by incubation with an HRP-conjugated anti-mouse IgG secondary antibody (1:3000 dilution in PBST). Detection of cytochrome c on blots was performed using enzyme-linked chemiluminescence (ECL, Amersham Pharmacia Biotech, Baie D'Urfe, PQ, Canada).

HeLa cytochrome c release was determined identically except that since HeLa are physically larger than Jurkat and since digitonin is a nonionic detergent requiring strict membrane to detergent ratios, only $2x10^6$ HeLa cells were used per sample.

2.3.5 Inhibitor Studies

Jurkat targets ($1x10^6$ cells/ml) were pre-treated for 30 minutes at 37°C with either zVAD-fmk or zDEVD-fmk at the concentrations indicated. Following this incubation, targets were treated with granzyme B and adenovirus or granzyme B and perforin as outlined in **section 2.3.1**. Alternatively, cells were treated with anti-human Fas IgM antibody (500 ng/ml) for 6 hours at 37°C. Apoptosis was determined as outlined **sections 2.3.2 and 2.3.3**, but with the following alterations. Rather than measuring both $DiOC_6(3)$ and HE on the same dot plot, the FL1 and FL2 channels were separated and $DiOC_6(3)$ and HE quantified on separate histograms, which facilitated quantification of the individual signals. For the experiments with cyclosporine A (CsA), Jurkats were treated with 30 μM protoporphyrin IX (PPIX) or granzyme B (1 μg/ml) and adenovirus (MOI=10pfu/cell) in the presence or absence of 100 μM CsA for two hours. The effects on $DiOC_6(3)$ loss were then determined by flow cytometry.

2.3.6 Caspase-3 Cleavage Assays

Supernatants were prepared as for the cytochrome c assay outlined in **section 2.3.4.** The supernatants were divided and half of each sample was prepared for SDS-PAGE analysis. Samples were resolved by SDS-PAGE (30 μl aliquots on 15% gels) and the separated proteins were transferred to nitrocellulose. Membranes were

blocked overnight in 5% milk proteins in PBST followed by a one hour incubation with a polyclonal anti-capsase-3 antibody (1:10,000 in 5% milk proteins in PBST). Following three five-minute washes in PBST, the membrane was incubated with a rabbit HRP-conjugated anti-mouse secondary antibody (1:6000 in 5% milk proteins in PBST). Proteins were visualized using ECL.

2.3.7 Measurement of Caspase-3 Activity

Caspase-3 activity was determined using a colourimetric assay with a DEVD-pNA substrate (Chemicon International Inc., Temecula, CA, USA). Samples were prepared as for **section 2.3.6**. The half of these samples not prepared for SDS-PAGE was utilized for the assessment of enzymatic activity. Cells ($5x10^5$) in 250 µl of ice-cold Lysis Buffer (50 mM Hepes, 10 mM $MgCl_2$ and 0.25%(v/v) Nonidet P-40) were incubated on ice for 10 minutes. Samples were centrifuged for one minute at 10 000xg at 4°C and the supernatants transferred to fresh tubes. The protein concentration of the supernatants was determined using a BCA kit (Pierce, Rockford, IL, USA). Samples (50 µl containing ~50-200 µg of total protein) were mixed with 50 µl of 2X Reaction buffer (100 mM Hepes, pH 7.5, 20% sucrose, 10 mM dithiothreitol and 0.2% (w/v) CHAPS). A DEVD-pNA substrate was added to each sample to a final concentration of 200 µM. Samples were then incubated at 37°C for 1-2 hours followed by absorbance measurements at 405 nm.

2.3.8 Subcellular Fractionation.

Subcellular fractionation of Jurkat cells was performed as described (Samali et al., 1999). Briefly, $1\text{-}6x10^8$ cells were collected for 10 minutes at 2000xg (3500 rpm in a Sorvall GSA rotor) and washed once with PBS. The pellet was washed once with buffer A (20 mM MOPS, pH 7.4, 100 mM sucrose, 1 mM EGTA) and then resuspended in a volume of buffer B (20 mM MOPS, pH 7.4, 100 mM sucrose, 1 mM EGTA, 5%

Percoll, and 191 µg/ml digitonin) giving a final cell density of $2x10^7$ cells/ml. Following a 15 minute incubation on ice with occasional stirring, the cells were spun at 2500xg (4500 rpm in a Sorvall SS-34 rotor) for 10 minutes at 4°C. The pellet, containing nuclei and cell debris, was discarded. The supernatant was further fractionated by centrifugation at 15 000xg (11 500 rpm in a Sorvall SS-34 rotor) for 15 minutes at 4°C. The mitochondrial fraction, a loose fluffy layer at the bottom of the tube, was collected, washed three times with buffer A and then resuspended in ~100-300 µl of buffer A. The supernatant (S-100) was spun at 100 000xg (39 000 rpm in a Beckman 70Ti rotor) for one hour at 4°C. The S-100 cytosolic fraction is herein referred to as the cytosol. Protein concentrations were determined using a BCA kit.

2.3.9 Immunodepletion of Bid from Jurkat cytosol.

Anti-human Bid antibodies (C-20 Santa Cruz) (or PBS alone for the mock control) were incubated in 325 µl PBS containing 4.5% protein A- and protein G-agarose (Amersham Pharmacia Biotech, Baie D'Urfé, Québec, Canada) for 3 hours at 4°C with rocking. The antibody-bound protein A/G beads were washed in buffer A and then incubated with 170 µg of Jurkat cytosol at 4°C for 18 hours with rocking. The agarose beads were then pelleted and the resulting supernatants labeled as C(-Bid) or C(mock) for Bid-depleted or mock-depleted cytosol respectively. Immunodepletion of Bid was verified by western blotting using a polyclonal rabbit anti-Bid antiserum.

2.3.10 In Vitro Assays with Purified Mitochondria

Purified mitochondria (10-20 µg) were mixed either with an equivalent amount of cytosol (10-20 µg) or an equivalent volume of buffer A alone as indicated (see **section 2.3.8** for the ingredients of buffer A). Granzyme B (0.5 µg) was added for 30 minutes at room temperature in the presence or absence of 100 µM zVAD-fmk. The mixtures were then spun for five minutes at 16 000xg (14 000 rpm in an Eppendorf

tabletop microfuge). The supernatants were transferred to fresh tubes and the pellets (mitochondria) were resuspended in a volume of buffer A equivalent to the initial sample volume. Pellets and supernatants were then mixed with 6x sodium dodecylsulphate (SDS) loading buffer, boiled for 10 minutes and loaded onto 15% SDS-polyacrylamide gels. Proteins were resolved at 200 V for ~50 minutes and subsequently transferred to nitrocellulose (Micron Separations Inc., Westborough, MA) at 150 mA for 1.25 hours in a semi-dry blotting apparatus (Tyler Instruments Inc., Edmonton, AB, Canada). Membranes were blocked overnight in 5% milk proteins (Carnation, Don Mills, Ontario, Canada) in PBST. Proteins were visualized with a monoclonal anti-human cytochrome c antibody (1:2000) followed by a goat anti-mouse HRP-conjugated secondary antibody (1:3000) followed by ECL. Immunoblotting for Bid and Bax was performed as for cytochrome c with the following modifications. Rabbit anti-mouse Bid (which cross-reacts with human) was used at 1:4000 to 1:8000. The goat anti-rabbit HRP-conjugated secondary was used at 1:20,000. Rabbit anti-human Bax was used at 1:400 to 1:1000.

2.3.11 Alkaline Extraction of Mitochondria.

Mitochondria were incubated under the conditions indicated. Following incubation, mitochondria were centrifuged at 16 000xg (14 000 rpm in an Eppendorf table-top microfuge) for 10 minutes at 4°C. The supernatants (pre-extraction supernatants) were removed and to them was added 6x SDS loading buffer followed by boiling for 10 minutes. The mitochondria were resuspended in 0.1 M Na_2CO_3 for 30 minutes on ice. After this incubation, the extracted mitochondria were centrifuged at 100 000xg (39 000 rpm in a Beckman Ti100.2 rotor) for 10 minutes at 4°C. The supernatants were discarded and the pellets resuspended in a volume of buffer A equivalent to the volume of the starting samples as well as 6x SDS loading buffer. Enough SDS loading buffer was added to ensure that the samples would sink into the

gel wells but not too much that the maximum allowable volume of the wells was exceeded. These samples were boiled for 10 minutes and the extracted pellets and pre-extraction supernatants were resolved on 15 % polyacrylamide gels followed by transfer to nitrocellulose. The blots were probed for Bax as outlined in **section 2.3.10.**

2.4 In Vivo Killing Assays

2.4.1 DNA fragmentation and Membrane Damage

Chromium (^{51}Cr; a measure of gross membrane damage) and [^{3}H]thymidine release (a measure of DNA fragmentation) assays have been described previously (Darmon et al., 1996). Jurkat targets were incubated with human CTL (hCTL) in the presence or absence of 5 mM EGTA. Percent death was calculated as: %death = 100 x (sample-spontaneous lysis)/(total-spontaneous lysis).

2.4.2 Measurement of $\Delta\Psi_m$ Loss in JpEGFP Targets

Measurement of $\Delta\Psi_m$ was performed as follows. JpEGFP cells (including JpEGFPneo and JpEGFPBcl-2 clones) were pre-loaded with the cationic lipophilic dye 2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide (DASPEI; Molecular Probes, Eugene, OR) at 50 nm for 15 minutes at 37°C. Dye-loaded cells were treated for 30 minutes at 37°C in the absence or presence of 50 or 100 μM zVAD-fmk. Following this incubation, 100 μl of hCTL was added to 100 μl of targets in a 96-well plate at effector to target ratios of 1:1, 0.5:1 and 0.25:1. The cell mixture was spun at 400xg for 5 minutes to ensure contact between targets and effectors and incubated at 37°C for up to three hours. Loss of $\Delta\Psi_m$ was monitored using flow cytometry by gating on the green fluorescent JpEGFP population (530 nm filter) and monitoring the loss of DASPEI fluorescence (580 nm filter) relative to DASPEI fluorescence of cells in the absence of hCTL. Loss of $\Delta\Psi_m$ was similarly determined for JpEGFP treated with granzyme B (1

μg/ml) and adenovirus (MOI=10pfu/cell) for up three hours at 37°C in the presence or absence of 100 μM zVAD-fmk or zDEVD-fmk.

2.4.3 Alkaline Extraction of Whole Cells.

Jurkat cells ($8x10^6$/sample) were treated as indicated. Following treatment, cells were washed once with PBS and then resuspended in 400 μl digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose and 191 μg/ml digitonin). Cells were incubated on ice for 15 minutes followed by centrifugation at 300xg (2000rpm in an Eppendorf microfuge) at 4°C. The pellet, containing nuclei and unbroken cells, was discarded. The supernatant was then centrifuged at 16 000xg (14 000 rpm in an Eppendorf microfuge) for 15 minutes at 4°C. The supernatant contained the cytosol. The pellet, containing mitochondria, was subjected to alkaline extraction with 400 μl of 0.1 M Na_2CO_3 for 30 minutes on ice followed by centrifugation at 100 000xg (39 000 rpm in a Beckman 100.2 rotor) for 10 minutes at 4 °C. The supernatant was discarded and the membrane pellets resuspended with 6x SDS loading buffer followed by boiling for 10 minutes. The pre-extraction supernatant (cytosol) and the extracted mitochondrial proteins were resolved by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting for Bax.

2.4.4 Densitometric Analysis.

Blots were scanned at a resolution of 400 pixels per inch using an Agfa Arcus II scanner with Agfa FotoLook Software (Version 3.0) and imported into Adobe Photoshop (version 5.5). The scanned images were saved as grayscale PICT files and imported into Image Guage (Version 3.0). An area of 84 square pixels was then placed about each band and the pixel density determined by the software. Values were plotted as pixel density per unit area and are displayed as arbitrary units.

Chapter 3

Caspase-Independent Granzyme B-Mediated Killing[1]

[1] A version of this chapter has been previously published: Jeffrey A. Heibein, Michele Barry, Bruce Motyka and R. Chris Bleackley (1999) Granzyme B-Induced Loss of Mitochondrial Inner Membrane Potential ($\Delta\Psi_m$) and Cytochrome c Release Are Caspase Independent, *Journal of Immunology*, **163**: 4683-4693. *Copyright 1999. The American Association of Immunologists.*

Chapter 3: Caspase-Independent Granzyme B-Mediated Killing

3.1 Introduction

Cytotoxic T lymphocytes (CTLs) are cells of the immune system that are responsible for the destruction of tumours, the elimination of cells that have been infected by virus, and in addition, also play roles in organ transplant rejection and autoimmunity (for reviews see (Atkinson and Bleackley, 1995; Darmon et al., 1999; Kägi et al., 1996; Shresta et al., 1998)). Once a target:CTL conjugate has formed, CTL-derived granules containing cytolytic proteins reorient to the sites of contact between the two cells (Yannelli et al., 1986). The granules are then exocytosed and their contents released into the intermembrane space (Kupfer et al., 1986). Among the granule components are a variety of proteins including granzymes, members of the serine protease family of enzymes. Another granule protein, perforin, facilitates entry of the granzymes into the target cell. Collectively, these proteins have been shown to induce morphological and biochemical features of apoptosis in the target cell (Atkinson and Bleackley, 1995; Berke, 1995; Darmon et al., 1999; Kägi et al., 1996; Shresta et al., 1998).

The onset of apoptosis leads to the systematic disassembly of the target resulting in cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. It is now widely accepted that the apoptotic events occurring within the target cell arise following the proteolytic activation by granzymes of endogenous cysteine proteases called caspases (for review see Earnshaw et al., 1999; Nicholson, 1999; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). Indeed, granzyme B, the most potent of the cytolytic granzymes, has been shown in vitro to cleave and thereby activate caspase-3 (CPP32/Apopain/Yama) (Darmon et al., 1996;

Darmon et al., 1995; Martin et al., 1996; Quan et al., 1996). In addition, the closely related caspase-7 (Mch3/CMH-1/ICE-LAP3) is activated (Chinnaiyan et al., 1996; Gu et al., 1996) along with caspase 6 (Mch2) (Orth et al., 1996; Srinivasula et al., 1996), caspase-8 (FLICE/MACH/Mch5) (Muzio et al., 1996), caspase-9 (ICE-LAP6/Mch6) (Duan et al., 1996; Srinivasula et al., 1996) and caspase-10 (Mch4) (Fernandes-Alnemri et al., 1996). Target cells treated with purified granzyme B and perforin contain processed caspase-1 (Shi et al., 1996), caspase-3 and caspase-7 (Chinnaiyan et al., 1996). Moreover, cleaved, active caspase-3 and -8 are found in target cells following exposure to allogeneic CTL (Atkinson et al., 1998; Darmon et al., 1996). The activation of caspase-3 has been linked with the onset of hallmark apoptotic events such as DNA fragmentation and membrane alterations through the cleavage of cellular substrates such as ICAD (DFF45; (Enari et al., 1998)) and gelsolin (Kothakota et al., 1997) as well as numerous others (Nicholson and Thornberry, 1997; Stroh and Schulze-Osthoff, 1998; Thornberry and Lazebnik, 1998). Thus, recent models of granule-dependent CTL-mediated killing suggested that apoptosis arising within targets was the direct result of granzyme B-mediated caspase-3 activation. While granzyme B likely initiates apoptosis via caspase-3 in many instances, there have been reports suggesting that granule-dependent killing may also occur through caspase-independent means (Sarin et al., 1998; Sarin et al., 1997)

Recently, mitochondria have been shown to undergo a number of profound changes early within the apoptotic program and appear to play a central role in apoptosis in a number of systems (for review see Green and Kroemer, 1998; Green, 1998; Green and Reed, 1998; Kroemer et al., 1997). The mitochondrial changes implicated include a disruption of electron transport and energy metabolism, alterations in the cellular redox potential, the production of reactive oxygen species (ROS), and a loss of the inner membrane transmembrane potential ($\Delta\Psi_m$), which may be

independent of or lead to the opening of permeability transition (PT) pores (for a review of the mitochondrial permeability transition, see (Zoratti and Szabò, 1995)). In addition a number of mitochondrial apoptogenic proteins including caspases-2, -3 and -9, apoptosis-inducting factor (AIF) and cytochrome c are released from the intermembrane space early during apoptosis in many systems (Kluck et al., 1997; Liu et al., 1996; Susin et al., 1999; Susin et al., 1996; Yang et al., 1997). AIF and cytochrome c have been implicated in the activation of caspase-3, linking mitochondrial events with caspase activation (Li et al., 1997; Liu et al., 1996; Susin et al., 1996; Zou et al., 1997). Current models of granule-mediated target cell apoptosis do not implicate the mitochondrial events outlined above. Indeed, the fact that granzyme B cleaves caspase-3 directly in vitro (Darmon et al., 1995; Martin et al., 1996; Quan et al., 1996) and that proteolytically activated caspase-3 is found in target cells exposed either to CTL or to purified granzyme B and perforin (Darmon et al., 1996; Darmon et al., 1995) even in the presence of zVAD-fmk (Atkinson et al., 1998) suggests that mitochondria are not required during caspase-dependent granule-mediated killing. However, it remained possible that mitochondria played a role in the overall demise of the cell, particularly in the caspase-independent pathway.

In order to address directly the involvement of mitochondria in the granzyme-mediated pathway, we examined the effect of granzyme B on mitochondrial function in both in vitro and in vivo systems. Two granzyme B-dependent in vitro assays were used. The first relied on perforin and the second utilized a replication deficient adenovirus to facilitate internalization of granzyme B (Froelich et al., 1996). Identical results were obtained with both in vitro systems. Jurkat cells induced to undergo apoptosis in response to granzyme B in vitro exhibit signs of $\Delta\Psi_m$ collapse, ROS production and cytochrome c release from mitochondria. Importantly, while DNA fragmentation and phosphatidylserine externalization are inhibited in the presence of

the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk), cytochrome c release and $\Delta\Psi_m$ loss are zVAD-fmk insensitive indicating that mitochondrial disruption in these cells is caspase-independent. In addition, Jurkats exposed to allogeneic CTL also exhibit $\Delta\Psi_m$ disruption which is zVAD-fmk insensitive. Thus we demonstrate for the first time that granzyme B-mediated death of Jurkat targets involves caspase-independent mitochondrial disruption.

3.2 Results

3.2.1 Jurkats Undergo Apoptosis in Response to Granzyme B and Perforin or Granzyme B and Adenovirus

Following the CTL-mediated induction of apoptosis in a target cell, several morphological and biochemical features become apparent. These features include DNA fragmentation (Wyllie, 1980) and membrane alterations such as phosphatidylserine externalization from the inner to the outer leaflet of the plasma membrane (Fadok et al., 1992; Martin et al., 1995). Cultured cells treated with granzyme B and perforin also exhibit these characteristic features of apoptosis (Chinnaiyan et al., 1996). In addition, replication deficient adenovirus has been shown to be a reliable substitute for perforin in that the virus facilitates internalization of granzyme B into target cells (Froelich et al., 1996; Pinkoski et al., 1998).

The human T cell lymphoma line Jurkat was treated with granzyme B and perforin or granzyme B and adenovirus for 2 hours at 37°C. The induction of apoptosis was then assessed by measuring DNA fragmentation using TUNEL and phosphatidylserine externalization using FITC-annexin V binding. The percent of apoptotic cells was then quantified using flow cytometric analysis by measuring the increases in TUNEL or FITC-annexin V fluorescence in apoptotic cells relative to controls. The data presented in **Figure 3-1** are a graphical representation of the flow

cytometry data for both TUNEL and FITC-annexin V binding. Significant levels of both DNA fragmentation (43%, 28%) and annexin V binding (41%, 27%) was seen when the targets were treated with granzyme B and adenovirus or perforin respectively. Cells in the absence of treatment (control) were approximately 5% TUNEL or FITC-annexin V positive. Likewise, the TUNEL or FITC-annexin V signals in Jurkats treated with granzyme B, or perforin, or adenovirus alone were less than 10%. Both DNA fragmentation and phosphatidylserine externalization were inhibited when granzyme B enzymatic activity was blocked with 3,4-dichloroisocoumarin (DCI; data not shown). Thus, targets treated with both enzymatically active granzyme B and perforin or active granzyme B and adenovirus displayed a 5-10-fold increase in both DNA fragmentation and phosphatidylserine externalization over untreated cells or cells treated with each component alone.

3.2.2 Cells Undergoing Apoptosis in Response to Granzyme B and Perforin/Adenovirus Experience $\Delta\Psi_m$ Loss and Generate ROS

The production of ROS and the loss of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$), have been identified as common early events occurring in apoptosis induced by a variety of stimuli (Green and Kroemer, 1998; Green and Reed, 1998; Kroemer et al., 1997). We monitored the integrity of the $\Delta\Psi_m$ during apoptosis induced by granzyme B and perforin, or granzyme B and adenovirus using the potential-sensitive dye $DiOC_6(3)$, which at low concentration targets to the negatively charged environment of the mitochondrial matrix in intact cells (Kroemer et al., 1997). During apoptosis, dissipation of the $\Delta\Psi_m$ leads to leakage of $DiOC_6(3)$ from the matrix, which can be measured by flow cytometry as a decrease in the fluorescence intensity of $DiOC_6(3)$ (Kroemer et al., 1997; Zoratti and Szabò, 1995). This is visualized as a shift in the dye-loaded population from the lower right ($DiOC_6(3)^+$) to the lower left

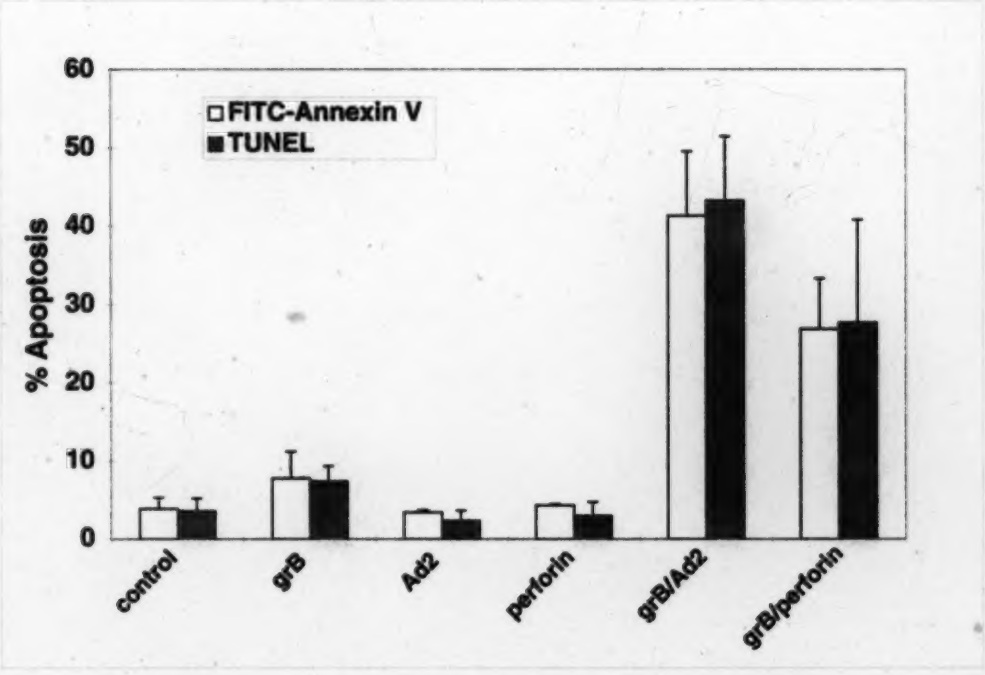


Figure 3-1 Granzyme B in combination with perforin or adenovirus causes apoptosis in Jurkats in vitro.

Jurkat cells were treated with granzyme B and perforin or granzyme B and adenovirus as outlined in **Chapter 2 (section 2.3.1)**. Following a 2 hour incubation at 37°C, 200 µl aliquots were removed and apoptosis assessed by TUNEL and by FITC-annexin V binding as outlined in **Chapter 2 (section 2.3.2)**. The number of apoptotic cells was quantified by flow cytometry. The means and standard deviations of at least three independent experiments are shown.

($DiOC_6(3)^-$) quadrant of a dot plot. ROS production within the cell can be measured simultaneously with $\Delta\Psi_m$ using hydroethidine (HE) which is oxidized to ethidium in the presence of ROS and exhibits red fluorescence following intercalation into cellular DNA. ROS production is visualized using flow cytometry as a shift from the lower left (HE^{low}) to the upper left (HE^{high}) quadrants of a dot plot.

In the absence of treatment (cells alone), $DiOC_6(3)$ fluorescence was apparent ($DiOC_6(3)^+$), indicating retention of the dye in mitochondria and an intact $\Delta\Psi_m$ (**Figure 3-2**). Jurkats treated with the protonophore mClCCP, a mitochondrial uncoupler (Heytler and Pritchard, 1962), exhibited a reduction in the retention of $DiOC_6(3)$ seen as a shift in the population from $DiOC_6(3)^+$ to $DiOC_6(3)^-$. This shift indicated a compromise in $\Delta\Psi_m$ integrity. In addition, there was an enhanced conversion of HE to ethidium (HE^{high} cells) indicative of ROS production in these cells. When Jurkats were incubated with granzyme B, perforin or adenovirus alone, neither $\Delta\Psi_m$ disruption nor ROS production was observed. However, when cells were treated with granzyme B and perforin or granzyme B and adenovirus together for 2 hours, $\Delta\Psi_m$ collapse and ROS production were apparent. As was seen for DNA fragmentation and phosphatidylserine externalization, no $\Delta\Psi_m$ loss or ROS production was observed when granzyme B enzymatic activity was inhibited with DCI (data not shown). Thus the combinations induce mitochondrial disruption and ROS production in Jurkat targets in vitro. Similar results have been seen for L1210 cells treated with granzyme B and perforin and EL4 cells treated with granzyme B and adenovirus (data not shown).

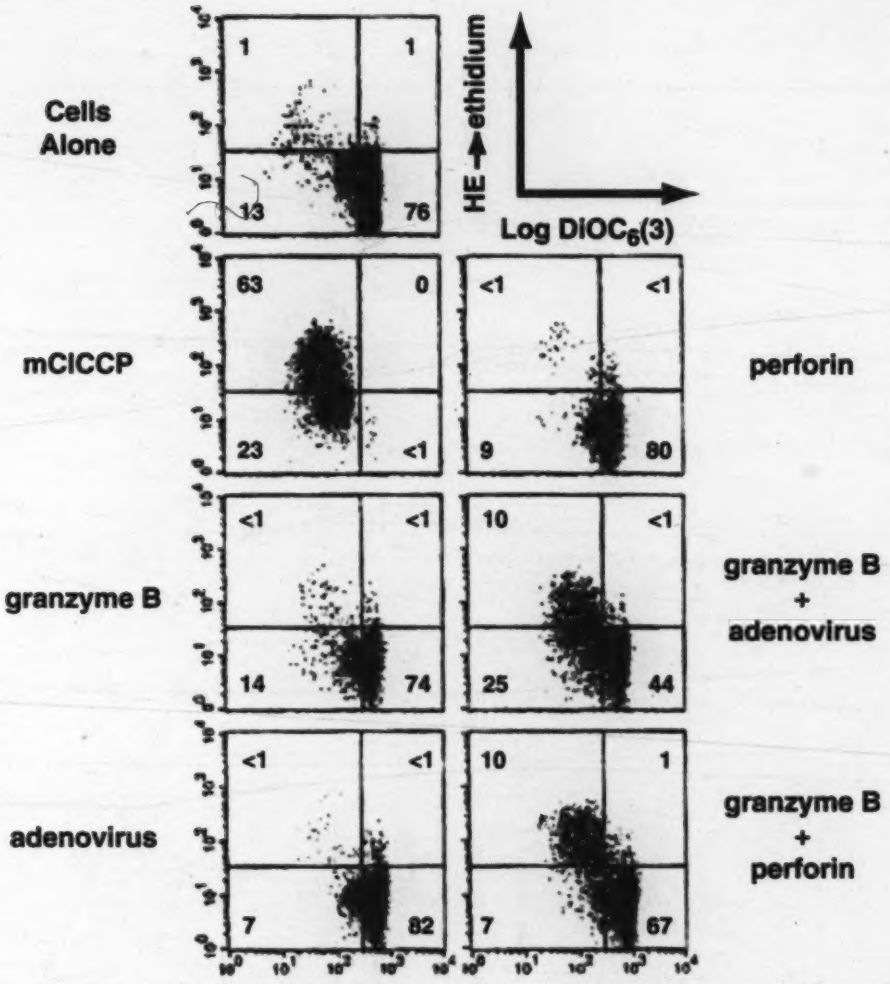


Figure 3-2 Granzyme B in combination with perforin or adenovirus causes $\Delta\Psi_m$ loss and ROS production in Jurkats in vitro.

Jurkat cells were treated with granzyme B and perforin or granzyme B and adenovirus as outlined in **Chapter 2 (section 2.3.1)**. $\Delta\Psi_m$ loss was determined using 40 nM $DiOC_6(3)$ and ROS production was assessed with 2 μM HE **(Chapter 2, section 2.3.3)**. The dyes were added together 15 minutes prior to the 2 hour endpoint of the assay. $\Delta\Psi_m$ loss and ROS production were then monitored by flow cytometric analysis. The data are representative of at least three independent experiments.

3.2.3 DNA Fragmentation and Phosphatidylserine Externalization Mediated Through the Granzyme B Pathway are Caspase-Dependent But $\Delta\Psi_m$ Collapse is Caspase-Independent

As a result of the dependence of the Fas system on caspases (Peter and Krammer, 1998), Fas-mediated apoptosis is exquisitely sensitive to peptide caspase inhibitors (Garcia-Calvo et al., 1998). When Jurkats were treated with anti-Fas antibodies for 6 hours, all measures of apoptosis examined, including DNA fragmentation, phosphatidylserine externalization, $\Delta\Psi_m$ collapse and ROS production were abrogated in the presence of the broad-spectrum caspase inhibitor zVAD-fmk at concentrations as low as 5 μM (**Figure 3-3A**). Here, $\Delta\Psi_m$ collapse and ROS production (HE conversion to ethidium) were quantified on separate dot histograms rather than on the same two-colour dot plots. zDEVD-fmk, an inhibitor primarily of caspase-3-like caspases, also completely abrogated all measured signs of apoptosis albeit at higher inhibitor concentrations (between 20-100 μM). Cells showed no signs of $\Delta\Psi_m$ loss or ROS production in the presence of either zVAD-fmk or zDEVD-fmk alone (data not shown).

When cells were treated with granzyme B and adenovirus in the presence zVAD-fmk or zDEVD-fmk, DNA fragmentation, phosphatidylserine externalization and ROS production were inhibited in a concentration dependent fashion (**Figure 3-3B**) as was seen for anti-Fas-treated cells (**Figure 3-3A**). While 20 μM zVAD-fmk or zDEVD-fmk were sufficient to abrogate DNA fragmentation, concentrations up to 100 μM were necessary to inhibit phosphatidylserine externalization and ROS production. Importantly, in these cells $\Delta\Psi_m$ loss was not prevented in the presence of 100 μM of either inhibitor. Similar results were observed when Jurkats were treated with granzyme B and perforin (data not shown). Thus, while the onset of classic apoptotic

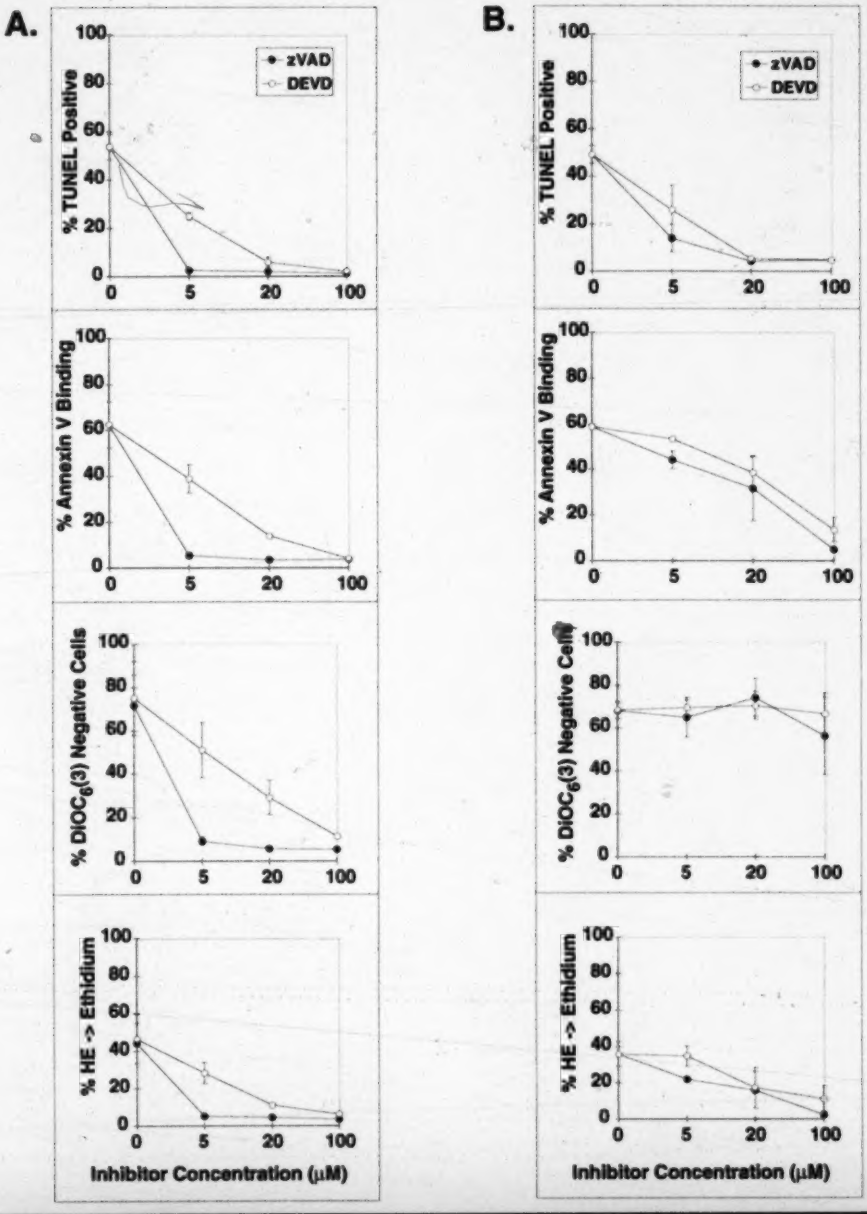


Figure 3-3 Anti-Fas antibody-induced $\Delta\Psi_m$ loss is caspase-dependent whereas that induced by granzyme B is not.

Jurkat targets were treated for 6 hours with anti-Fas antibody (**A**) or for 2 hours with granzyme B and adenovirus (**B**) in the presence or absence of zVAD-fmk or zDEVD-fmk at the concentrations indicated. Apoptosis was monitored using TUNEL, FITC-annexin V binding, $DiOC_6(3)$ retention and conversion of HE to ethidium as outlined in **Chapter 2** (**sections 2.3.2** and **2.3.3**). Means and standard deviations represent at least three independent experiments.

morphology induced by granzyme B and adenovirus was caspase-dependent in a two-hour assay, $\Delta\Psi_m$ collapse was caspase-independent.

3.2.4 Cyclosporine A Does Not Block Granzyme B-Mediated $\Delta\Psi_m$ Loss

Granzyme B-dependent killing involves a decrease in $\Delta\Psi_m$ which occurs through a caspase-independent process (**Figure 3-3B**). The immunosupressive agent cyclosporine A (CsA) has been shown to inhibit the $\Delta\Psi_m$ loss caused by PT pore opening in many but not all systems (Zoratti and Szabò, 1995). One system in which CsA has been shown to prevent $\Delta\Psi_m$ loss involves treatment with the PT pore-inducer protoporphyrin IX (PPIX), (Marchetti et al., 1996). As was shown previously (Marchetti et al., 1996), cells treated with mClCCP or PPIX experience a loss of $\Delta\Psi_m$ as measured by a decrease in $DiOC_6(3)$ fluorescence (**Figure 3-4**). The PPIX-induced $\Delta\Psi_m$ loss was prevented in the presence of 100 µM CsA. By contrast, CsA did not prevent the loss of $DiOC_6(3)$ from cells treated with granzyme B and adenovirus suggesting the granzyme B-dependent $\Delta\Psi_m$ loss observed occurred through a CsA-insensitive mechanism.

3.2.5 Jurkat Cells Exposed to Human CTL Exhibit $\Delta\Psi_m$ Collapse

It was necessary to confirm that the results observed in Jurkats treated with granzyme B and perforin, or granzyme B and adenovirus in vitro were also occurring in vivo in response to allogeneic CTL. CTL have been shown to kill targets predominantly through two mechanisms, the granule-mediated and Fas pathways (Atkinson and Bleackley, 1995; Shresta et al., 1998). In order to determine which pathway prevailed in our in vivo system, we relied on the differential sensitivity of the two pathways to Ca^{2+} chelation, the granule-mediated pathway being Ca^{2+}-dependent. Jurkat targets were exposed to human CTL (hCTL) at a 2:1 effector-to-target ratio in the absence or presence of 5 mM EGTA (**Figure 3-5A**). The amount of [^{3}H]thymidine release, a

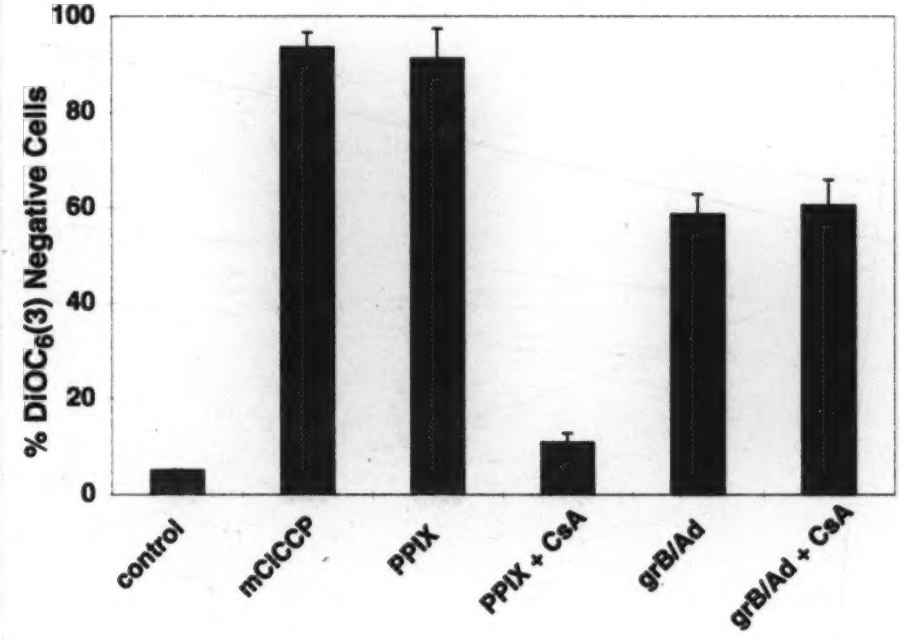


Figure 3-4 Cyclosporine does not inhibit $\Delta\Psi_m$ loss mediated by granzyme B and adenovirus.

Jurkats were treated with either PPIX (30 μM) or granzyme B and adenovirus for 2 hours in the presence or absence of 100 μM CsA. Loss of $\Delta\Psi_m$ was measured using 40 nm $DiOC_6(3)$ and flow cytometry as described in **Chapter 2 (section 2.3.3)**. Means and standard deviations of at least two independent experiments shown.

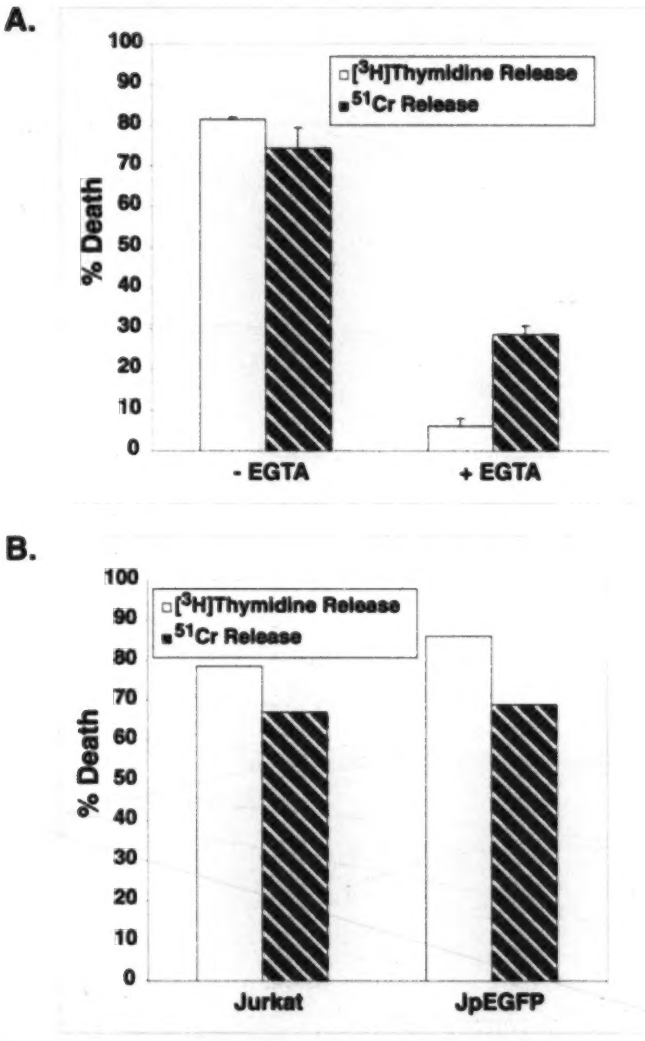


Figure 3-5 Human CTL induce DNA fragmentation and membrane damage in Jurkats.

DNA fragmentation was measured using $[^3H]$thymidine release following 2 hours at 37°C. Membrane damage was assessed by ^{51}Cr release after 4 hours at 37°C. **A.** Jurkats are killed by human CTL (hCTL) primarily through granule-mediated cytotoxicity. Jurkats were incubated with hCTL at an effector-to-target ratio of 2:1 in the absence or presence of 5 mM EGTA. **B.** JpEGFP targets behave like Jurkat targets. GFP-expressing JpEGFP targets were incubated with hCTL at an effector:target ratio of 2:1 and $[^3H]$thymidine release and ^{51}Cr release were determined as outlined above. The mean and standard deviation of triplicate samples are shown.

measure of DNA fragmentation, decreased dramatically from 90% to less than 20% in the presence of EGTA. Similar results were observed for ^{51}Cr release, a measure of plasma membrane damage. Furthermore, when Jurkats were pre-treated with a blocking anti-Fas IgG antibody to prevent Fas-mediated killing, no impairment of either DNA fragmentation or ^{51}Cr release was observed relative to control targets in the absence of antibody (data not shown). Thus we conclude that the cytolysis of Jurkats mediated by allogeneic CTL in our system involves predominantly the granule-dependent pathway.

To be sure that the $\Delta\Psi_m$ disruption seen in cells treated in vitro with granzyme B and perforin, or granzyme B and adenovirus was occurring in vivo, studies were undertaken in which Jurkats were pre-loaded with $DiOC_6(3)$ and then incubated with hCTL at a fixed effector-to-target ratio. Jurkats were loaded prior to, rather than following, experimentation in an effort to differentiate the targets from the effectors. However, using these means, it proved difficult to distinguish targets from effectors by flow cytometry. To alleviate this problem, Jurkat targets were generated which expressed a green fluorescent protein (JpEGFP; see **Chapter 2 section 2.1.1.4**). The green fluorescence of these cells allowed them to be distinguished from effectors by flow cytometry. By gating on green-fluorescent cells and by utilizing a red-fluorescent potential-sensitive dye, DASPEI, which has been used previously to monitor mitochondrial membrane potential (Marshall et al., 1992; McCormick, 1990), it was possible to examine mitochondrial events in these cells. JpEGFP targets were pre-treated with DASPEI prior to addition of hCTL to further ensure that the signals observed were coming only from the targets. Using in vitro experiments, the disruption of $\Delta\Psi_m$ in targets treated with granzyme B and adenovirus appears the same whether examined with $DiOC_6(3)$ or DASPEI and whether the dyes are added prior to or following the addition of the apoptotic stimulus (data not shown). Thus we are confident

that pre-loading JpEGFP targets with DASPEI is a viable technique for examining $\Delta\Psi_m$ changes in vivo.

To confirm that the JpEGFP targets responded to allogeneic CTL in the same fashion as Jurkats, JpEGFP were exposed to hCTL at an effector-to-target ratio of 2:1 and analyzed for [^{3}H]thymidine release and ^{51}Cr release (**Figure 3-5B**). The data show clearly that JpEGFP cells undergo apoptosis in response to hCTL, displaying levels of DNA fragmentation and membrane disruption comparable to parent Jurkat targets. The JpEGFP clones were also sensitive to EGTA in a fashion similar to parent Jurkats (data not shown).

Having determined that JpEGFP cells underwent apoptosis in response to hCTL attack, we wanted to know if they also experienced the loss in $\Delta\Psi_m$ seen when Jurkats were treated with granzyme B and perforin or granzyme B and adenovirus (**Figure 3-2**). To this end, JpEGFP cells were pre-loaded with DASPEI and then exposed to hCTL for three hours at the effector to target ratios indicated (**Figure 3-6A**). $\Delta\Psi_m$ disruption was observed in the green-fluorescent target population as a loss in DASPEI fluorescence seen at effector-to-target ratios as low as 0.25:1. As observed previously (**Figure 3-3**), loss of $\Delta\Psi_m$ was not inhibited by zVAD-fmk. At a zVAD-fmk concentration of 100 μM, $\Delta\Psi_m$ loss was largely resistant (70%) at all effector-to-target ratios tested. It is possible that the small amount of sensitivity (30%) observed was due to the small amount of Ca^{2+}-independent killing seen in the presence of EGTA (**Figure 3-5A**).

Similar to the results with hCTL, $\Delta\Psi_m$ loss was again largely resistant to zVAD-fmk (~80%) in DASPEI-loaded JpEGFP targets treated with granzyme B and adenovirus (**Figure 3-6B**). Hence, in our in vivo system, $\Delta\Psi_m$ loss mediated through the granzyme pathway was predominantly caspase-independent.

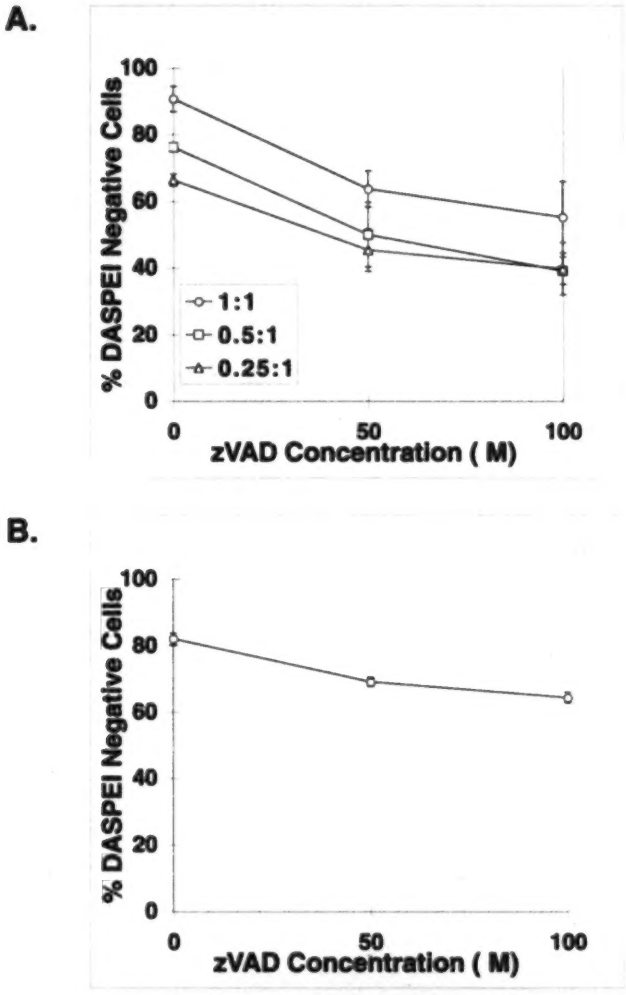


Figure 3-6 $\Delta\Psi_m$ loss in JpEGFP targets in response to the granzyme B pathway is caspase-independent.

A. $\Delta\Psi_m$ loss in DASPEI-loaded JpEGFPs treated with hCTL is not inhibited in the presence of zVAD-fmk. JpEGFP targets were pre-loaded with 50 nM DASPEI in serum-free RPMI-1640 for 15 minutes at 37°C. The cells were washed and then treated for 30 minutes with zVAD-fmk at the indicated concentrations. Following this incubation, JpEGFPs were exposed to hCTL at effector-to-target ratios of 1:1, 0.5:1, and 0.25:1. The cells were incubated at 37°C for 3 hours and the $\Delta\Psi_m$ of the dye-loaded population was monitored by flow cytometric analysis. **B.** Granzyme B/adenovirus-induced loss of $\Delta\Psi_m$ in DASPEI-loaded JpEGFP targets is caspase-independent. DASPEI-loaded JpEGFP targets were treated with granzyme B and adenovirus for 3 hours at 37°C in the presence or absence of zVAD-fmk at the concentrations indicated. Means and standard deviations of triplicate samples shown.

3.2.6 Cytochrome c Release From Mitochondria During Granzyme B-Mediated Apoptosis Is Caspase-Independent

The release of cytochrome c from mitochondria appears to be an early event during apoptosis induced by a variety of stimuli (Kluck et al., 1997; Yang et al., 1997). Cytosolic cytochrome c has been shown to promote the formation of a complex with Apaf-1 and pro-caspase-9 through a process dependent on dATP/ATP (Liu et al., 1996; Zou et al., 1997). The formation of this apoptosome facilitates cleavage and activation of caspase-9, which then proteolytically activates caspase-3 (Li et al., 1997). Cytochrome c release has been shown to occur in Jurkats following treatment with anti-Fas antibodies (reviewed in (Green, 1998; Peter and Krammer, 1998; Reed, 1997)). Since Jurkats are type II cells (Scaffidi et al., 1998), cytochrome c release and subsequent apoptosis mediated through the Fas pathway is exquisitely sensitive to the caspase inhibitor zVAD-fmk. To confirm the caspase-dependence of Fas-mediated cytochrome c release in our system, Jurkat targets were treated with anti-Fas antibodies for up to 6 hours in the absence and presence of 100 μM zVAD-fmk. Following treatment, cells were collected by centrifugation and the pellets were resuspended in digitonin lysis buffer. At low concentrations, the nonionic detergent digitonin selectively renders the plasma membrane permeable while leaving other organelles intact (Adam et al., 1990). Following permeablization, cells were centrifuged to separate the membrane fraction (including mitochondria) from the cytosol fraction. Proteins of both supernatants (cytosol) and pellets (membranes) were resolved on 15% polyacrylamide gels followed by transfer to nitrocellulose membranes. Blots were probed with a monoclonal anti-cytochrome c antibody. As a control for the presence of cytochrome c, purified rat mitochondria (Rat Mito.) were also loaded onto these gels (**Figure 3-7**). The data indicate that in the absence of zVAD-fmk, cytochrome c translocates from the membrane fraction to the cytosol (**Figure 3-7**, lanes 10-12). This

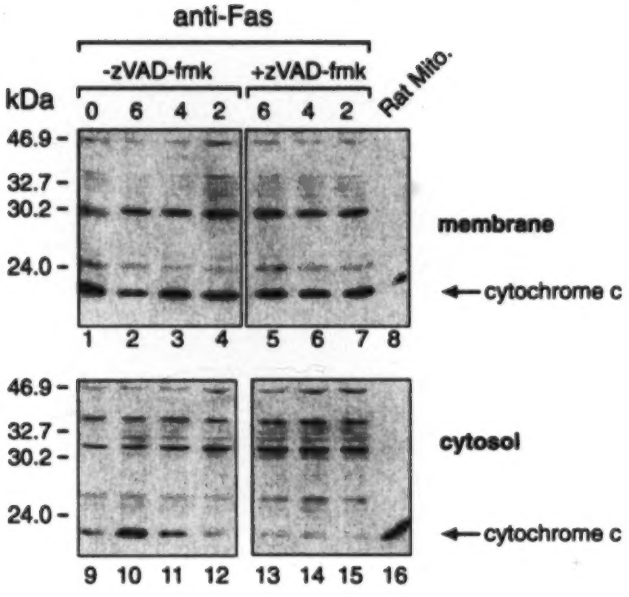


Figure 3-7 Anti-Fas antibody-induced cytochrome c release is caspase-dependent.

Jurkats were treated with anti-Fas antibody (500 ng/ml) for 0, 2, 4 and 6 hours at 37°C in the presence or absence of 100 μM zVAD-fmk. Cells were washed in PBS and resuspended in digitonin lysis buffer for 5 minutes on ice followed by centrifugation. 25 μl of pellets and 50 μl of supernatants in SDS-loading buffer were loaded onto 15% gels. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes and probed with a monoclonal anti-cytochrome c antibody (1:2000). Following the addition of an anti-mouse HRP secondary antibody, proteins were detected using ECL. Membrane (pellet) and cytosol (supernatant) fractions shown. Data representative of two independent experiments.

translocation is apparent within 4 hours following the initiation of treatment (**Figure 3-7**, lane 11). However, in the presence of zVAD-fmk, cytochrome c release is completely abrogated even at 6 hours following treatment (**Figure 3-7**, lanes 13-15). Thus, as has been previously reported (Peter and Krammer, 1998), Fas-induced cytochrome c release is caspase-dependent in Jurkat targets.

Since we have shown that granzyme B in combination with perforin or adenovirus can induce $\Delta\Psi_m$ loss suggesting possible effects of granzyme B on mitochondria (**Figure 3-2**), we were interested to know if release of cytochrome c was also occurring in granzyme B-treated Jurkat targets. Cytochrome c release from the membrane fraction (containing mitochondria) to the cytosol did occur in Jurkats in response to granzyme B and adenovirus (**Figure 3-8A**, lanes 13-17). Cytochrome c was detected in the cytosol as early as 30 minutes following administration of granzyme B and adenovirus (**Figure 3-8A**, lane 15). Cytochrome c was not released in response to granzyme B or adenovirus alone (**Figure 3-8A**, lanes 18 and 19 respectively). Importantly, in contrast to anti-Fas-treated cells, granzyme B/adenovirus-mediated cytochrome c release also occurred in the presence of 100 μM zVAD-fmk (**Figure 3-8A**, lanes 20-24). Identical results were observed in HeLa cells treated with granzyme B and adenovirus (**Figure 3-8B**). Cytochrome c release did not occur when granzyme B enzymatic activity was inhibited with DCI (data not shown). Therefore, the granzyme B-dependent pathway can induce cytochrome c loss from mitochondria through a mechanism that required granzyme B enzymatic activity but did not rely upon caspases.

3.2.7 Granzyme B-Mediated Membrane Damage is Caspase-Independent

Jurkats treated with granzyme B and adenovirus release cytochrome c from their mitochondria and lose $\Delta\Psi_m$ in a caspase-independent fashion despite the

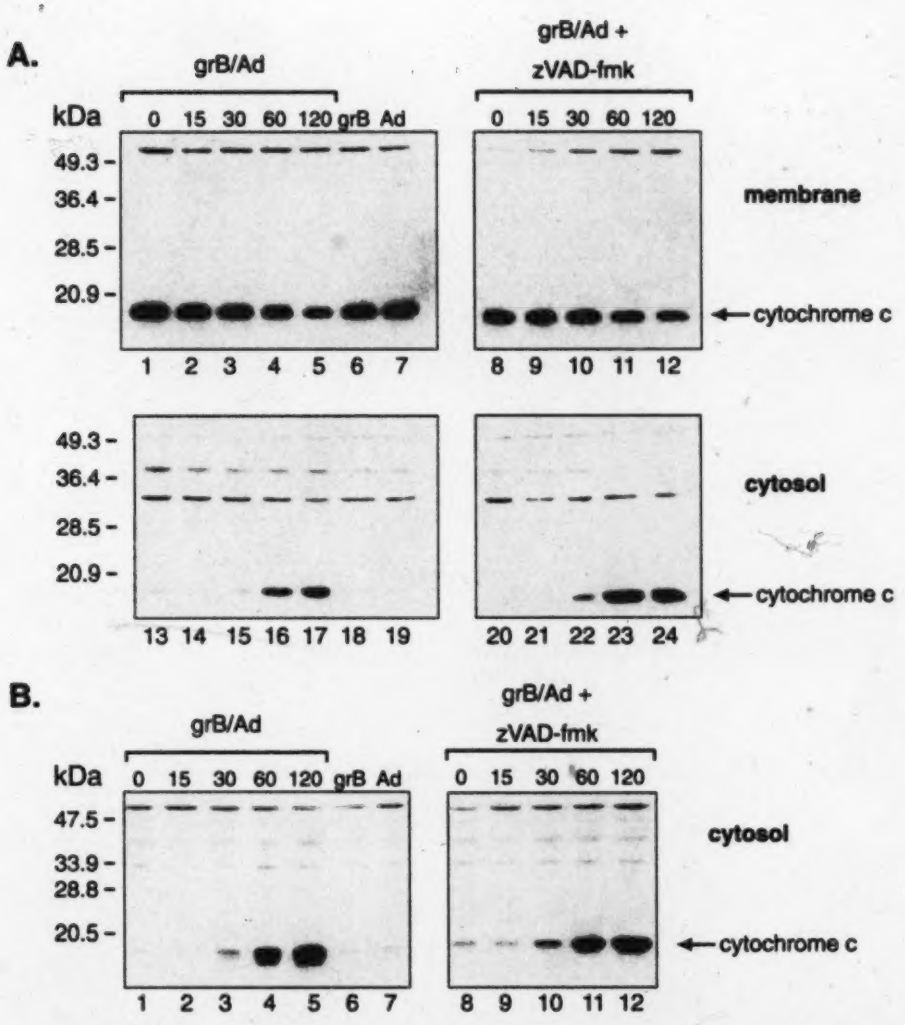


Figure 3-8 Cytochrome c release induced by granzyme B and adenovirus is caspase-independent.

A. Jurkats were treated with granzyme B (1μg/ml) and adenovirus (MOI=10pfu/ml) for 0, 15, 30, 60, and 120 minutes at 37°C and processed as outlined for **Figure 3-7**. **B.** HeLa cells were treated with granzyme B and adenovirus as for **A.** Data representative of three independent experiments.

absence of an apoptotic morphology at early times following treatment (**Figures 3-3B and 3-8A**). To investigate the long-term consequences of caspase inhibition following granzyme B treatment, Jurkats were treated with granzyme B and adenovirus for 24 hours in the presence or absence of either zVAD-fmk or zDEVD-fmk (**Figure 3-9**). At the indicated times, DNA fragmentation was assessed by [^{3}H]thymidine release and membrane damage was measured by ^{51}Cr release. In the presence of zVAD-fmk or zDEVD-fmk, granzyme B-mediated DNA fragmentation was blocked (~0%) even at 24 hours following initial treatment (**Figure 3-9A**). However, membrane damage, while initially diminished in the presence of the caspase inhibitors, reached similar levels by 24 hours post-treatment (~30% in the absence and presence of zVAD-fmk and ~20% in the presence of zDEVD-fmk) indicating that caspases are not required to elicit membrane damage. Importantly, in the Fas system on the other hand, both DNA fragmentation and membrane damage were blocked in the presence of the caspase inhibitors and were therefore dependent upon caspases (**Figure 3-9B**). Thus, cells treated with granzyme B and adenovirus for longer periods of time suffered membrane damage that was induced through a caspase-independent mechanism.

3.3 Discussion

Models of granzyme-dependent killing have thus far involved a direct activation of caspase-3 by granzyme B. While this likely occurs in many instances, there have been reports that granule-dependent killing can proceed in the absence of caspases (Sarin et al., 1998; Sarin et al., 1997).

We confirmed that apoptosis was occurring in our in vitro system of CTL-mediated killing by measuring DNA fragmentation, a hallmark of apoptosis (Wyllie, 1980), using TUNEL. Jurkat targets treated with granzyme B and perforin or granzyme B and adenovirus for 2 hours showed a 5-10-fold increase in DNA fragmentation over untreated cells or cells treated with granzyme B, perforin or adenovirus alone (**Figure**

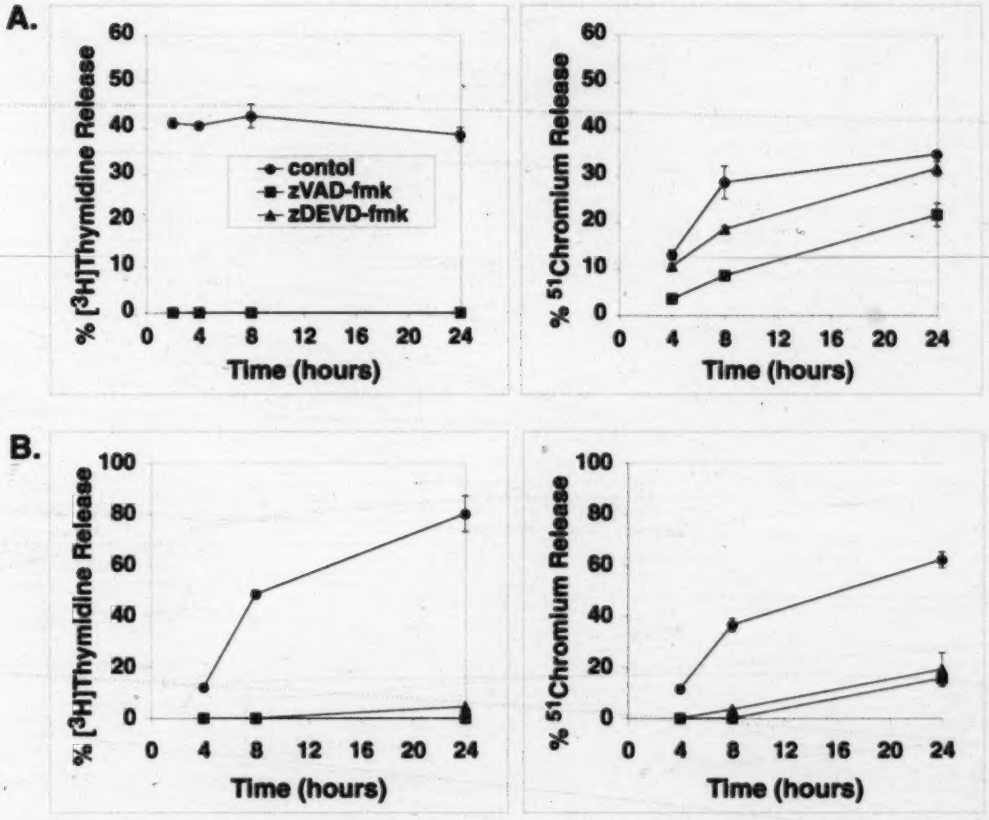


Figure 3-9 Granzyme B-induced DNA fragmentation is caspase-dependent whereas membrane damage is caspase-independent.

A. Jurkats were incubated with granzyme B and adenovirus in the presence or absence of 100 µM zVAD-fmk or zDEVD-fmk for 24 hours at 37°C. DNA fragmentation was determined by [^{3}H]thymidine release and membrane damage was assessed by ^{51}Cr release. **B.** Jurkats were incubated with anti-Fas antibodies in the presence or absence of 100 µM zVAD-fmk or zDEVD-fmk for 24 hours at 37°C. DNA fragmentation and membrane damage determined as for **A.** The means and standard deviations of two independent experiments are shown.

3-1). Granzyme B-mediated DNA fragmentation was absolutely dependent upon caspases as fragmentation was abrogated in the presence of either the broad-spectrum caspase inhibitor zVAD-fmk or the inhibitor of caspase-3-like caspases, zDEVD-fmk (**Figure 3-3B**). Either inhibitor at concentrations as low as 20 μM was sufficient to completely block DNA fragmentation. The ability of such low inhibitor concentrations to mediate such profound inhibition was similar to the effects of these inhibitors on DNA fragmentation seen when cells were treated with anti-Fas antibodies (**Figure 3-3A**); the Fas pathway is known to be absolutely dependent upon caspases (Peter and Krammer, 1998).

As a second confirmation of apoptosis in our system, we examined the extent of phosphatidylserine externalization from the inner to the outer leaflet of the plasma membrane. As seen for DNA fragmentation, granzyme B in combination with perforin or adenovirus induced an approximately 10-fold increase in phosphatidylserine externalization over cells treated with any of the components alone (**Figure 3-1**). Similar to their effects on granzyme B-induced DNA fragmentation, the caspase inhibitors zVAD-fmk and zDEVD-fmk were also able to prevent phosphatidylserine externalization in response to granzyme B and adenovirus (**Figure 3-3B**) or granzyme B and perforin (data not shown). However, concentrations up to 100 μM of either inhibitor were required in order to mediate this inhibition completely. This contrasts with the Fas system in which lower concentrations of either zVAD-fmk (5 μM) or zDEVD-fmk (20 μM) were sufficient to block phosphatidylserine externalization (**Figure 3-3A**). These differences may reflect the dependence of the Fas system on caspase-8 at the most proximal step of activation (Peter and Krammer, 1998) where zVAD-fmk would block apoptotic signaling at the plasma membrane. Peptide inhibitors containing the sequence DEVD are good inhibitors of caspase-3-like enzymes but are also reasonably good at inhibiting caspase-8 (Garcia-Calvo et al., 1998). Thus the

substantial inhibition of DNA fragmentation and phosphatidylserine externalization observed in the presence of zVAD-fmk likely points to caspase-8 inhibition at the initiation stage when little caspase activation has occurred. In contrast, the results with zDEVD-fmk may represent inhibition of caspase-3-like enzymes in the effector stages where more caspases may already have been activated, thereby requiring more zDEVD-fmk to achieve comparable inhibition to zVAD-fmk.

In the granzyme B pathway, the pronounced inhibition of DNA fragmentation by either zVAD-fmk or zDEVD-fmk even at times up to 24 hours following treatment (**Figures 3-3B** and **3-9**) points towards the dependence of DNA fragmentation in this system on caspase-3-like enzymes. Since higher concentrations of either inhibitor are required to block phosphatidylserine externalization in the granzyme B pathway, it is possible that a wider variety of caspases are required to induce membrane alterations through granzyme B-dependent events or that caspase-independent mechanisms are initiated. Caspase-independent phosphatidylserine externalization and α-fodrin cleavage in response to tumor necrosis factor (TNF) have been described (Vanags et al., 1996). Indeed, the fact that cells appear to have lost membrane integrity 24 hours following granzyme B treatment in the presence of either zVAD-fmk or zDEVD-fmk (**Figure 3-9A**) suggests that caspases are not required for the ultimate demise of the cell. Our results support the view of Henkart *et al.*, that killing by the granule pathway occurs through caspase-dependent and independent pathways (Sarin et al., 1998; Sarin et al., 1997). It appears that in particular, DNA fragmentation is sensitive to caspase inhibition. In contrast, membrane damage slows but still continues in the presence of either zVAD-fmk or zDEVD-fmk. It is tempting to speculate that the caspase-independent mitochondrial effects observed explain this necrotic form of death and may be responsible for cell death even in the presence of biological caspase

inhibitors such as the inhibitor of apoptosis proteins (IAPs) (Deveraux and Reed, 1999).

Events such as the loss of the mitochondrial inner transmembrane potential ($\Delta\Psi_m$), the release of mitochondrial cytochrome c, and in some cases, the production of reactive oxygen species (ROS), have been linked with the onset of apoptosis induced by a variety of stimuli. These stimuli include Fas cross-linking, TNF, glucocorticoids and a number of chemotherapeutic agents (Green and Kroemer, 1998; Green and Reed, 1998; Kroemer et al., 1997; Reed, 1997). We investigated the possible role of mitochondria in apoptosis induced through the granzyme B pathway and we observed that granzyme B induced a decrease in the $\Delta\Psi_m$ only when administered in conjunction with either perforin or adenovirus (**Figure 3-2**). When Jurkats were treated with hCTL or with granzyme B and adenovirus in the presence of either zVAD-fmk or zDEVD-fmk, $\Delta\Psi_m$ loss was not prevented (**Figures 3-3B** and **3-6**) suggesting that granzyme B-mediated events at the mitochondria occur through a caspase-independent mechanism. In this respect, our data supports those recently published by MacDonald *et al.* (MacDonald et al., 1999). This latter study used a single in vitro system based on perforin and granzyme B. Our present data extend this work to include a second in vitro system and most importantly, intact CTL. Thus the results of our in vitro studies were confirmed in a physiologically relevant model of killing where CTL induced death in targets at low effector-to-target ratios. There are, however, some differences between our conclusions and those of the earlier publication.

Whereas we found that both $\Delta\Psi_m$ collapse and release of cytochrome c occurred in the presence of zVAD-fmk (**Figures 3-3A, 3-8A** and **3-8B**), MacDonald *et al.* reported that cytochrome c release was caspase-dependent. Our experiments were performed primarily in Jurkats, however, we obtained identical results in HeLa, the

same cells used in the other study. It is possible that the explanation for the discrepancy lies in a difference in experimental design. The concentrations of granzyme B used were similar but it is difficult to rule out differences in the specific activities of the two enzymes. In addition, we used human granzyme B on human targets whereas the other group utilized a rat enzyme. To our knowledge, a direct comparison of the caspases activated by granzyme B between species has not been published. Nevertheless, it has been reported that murine granzyme B cleaves only a limited number of caspases and this contrasts with the variety of caspases activated by human granzyme B (Vande Craen et al., 1997). It is noteworthy in this regard that MacDonald *et al.* were able to block the initial cleavage of caspase-3 to p20 with zVAD-fmk in cells treated with granzyme B and perforin. This suggests that caspase-3 activation by granzyme B requires another caspase. In direct contradiction, we have clear evidence that, in both our *in vitro* and whole cell killing systems, this cleavage event is caspase independent (Atkinson et al., 1998).

Another variable between the two experimental strategies could be the role of perforin. In the other study, granzyme-independent effects were clearly evident. In our experiments, we used sublytic doses of perforin that gave no evidence of $\Delta\Psi_m$ collapse, ROS production or cytochrome c release in the absence of enzymatically active granzyme B. Moreover, we have never observed $\Delta\Psi_m$ loss in cells using concentrations of adenovirus greater than ten times those used for the experiments described here (Heibein *et al.*, unpublished observations). It may be that we are providing a milder, granzyme B-dependent stimulus to our target cell mitochondria. Indeed, at two hours we still see a considerable amount of cytochrome c in the mitochondria whereas MacDonald *et al.* completely depleted all mitochondrial cytochrome c at this time point. Despite the fact that our in vitro evidence strongly suggests that perforin by itself has no effect on mitochondria, we cannot exclude the

possibility that perforin may be having some effect when Jurkat targets are exposed to human CTL. At the sites of contact between target and CTL, the local concentration of perforin may be sufficiently higher than perforin added exogenously and therefore may be able to induce mitochondrial alterations. The differences between our two systems are intriguing and further studies to establish the basis of the discrepancies may be very informative for elucidating events in vivo.

Cytochrome c, once released from mitochondria, is believed to form a complex with Apaf-1 and pro-caspase-9. This 'apoptosome' then mediates activation of caspase-3 through a mechanism dependent on dATP/ATP. We do not have any direct evidence that cytochrome c released during granzyme B-mediated apoptosis forms a complex with Apaf-1 and caspase-9. Nevertheless, such an interaction has been described for Jurkats in response to the Fas pathway (Scaffidi et al., 1998), and so it is not unreasonable to assume that such a process may occur in these cells in response to granzyme B. Since granzyme B is capable of directly activating caspase-3 in intact cells (Atkinson et al., 1998; Darmon et al., 1996; Darmon et al., 1995; Martin et al., 1996; Quan et al., 1996), one possible role for the Apaf-1/cytochrome c/pro-caspase-9 complex is to amplify the cascade. Indeed, the role of the mitochondrion as an accelerator of the apoptotic response during granzyme B-dependent killing in a cell free system has recently been described (MacDonald et al., 1999). The results reported here indicate that in the granzyme pathway, only events downstream of mitochondria are sensitive to zVAD-fmk. This confirms our earlier report that initial caspase-3 activation is independent of any other caspase activity (Atkinson et al., 1998).

In some cases, $\Delta\Psi_m$ loss has been shown to result from or to induce the opening of permeability transition (PT) pores in the inner mitochondrial membrane (Zoratti and Szabò, 1995). PT pore opening induced by a variety of chemical and

physiological agents is inhibited in many, but not all instances, by the immunosupressive agent cyclosporine A (CsA) (Zoratti and Szabò, 1995). Jurkats were treated with PPIX, a ligand of the benzodiazapine receptor (Verma et al., 1988). The benzodiazapine receptor is a protein believed to be a constituent of the PT pore and PPIX has been shown to induce PT pore opening with the subsequent loss of $\Delta\Psi_m$ in thymocytes (Marchetti et al., 1996). Our results in Jurkats confirmed those previously observed in thymocytes in that PPIX was able to induce $\Delta\Psi_m$ loss in Jurkats in a CsA-inhibited fashion (**Figure 3-4**). By contrast, $\Delta\Psi_m$ loss induced by granzyme B and adenovirus was not inhibited in the presence of CsA. It is possible that the granzyme B pathway induces PT pore opening through a CsA-insensitive mechanism. Alternatively, it is likely that the $\Delta\Psi_m$ loss observed results not from PT pore opening, but rather, some other mechanism such as the loss of cytochrome c interfering with electron transport and oxidative phosphorylation which would disrupt the ability of mitochondria to maintain an intact $\Delta\Psi_m$.

The production of ROS has been shown to accompany apoptosis-induced $\Delta\Psi_m$ collapse (Zamzami et al., 1995) and in some instances, has been shown to facilitate PT pore opening (Green and Kroemer, 1998). In other cases, ROS production occurs well after classical apoptotic morphology is evident suggesting that ROS in these systems may arise as a consequence of $\Delta\Psi_m$ loss and the resultant uncoupling of electron transport and oxidative phosphorylation (Green and Reed, 1998). ROS production was observed in our Jurkat system in response to granzyme B and perforin or granzyme B and adenovirus (**Figure 3-2**). However, ROS production decreased in the presence of zVAD-fmk or zDEVD-fmk suggesting that ROS production was in some way dependent upon caspase activation (**Figure 3-3B**). Since ROS production was caspase-dependent while $\Delta\Psi_m$ loss was caspase-independent, it is unlikely that ROS production in the granzyme B system is the cause of $\Delta\Psi_m$ loss. Rather, ROS

production is likely a consequence of caspase activation and the subsequent activation by caspases of downstream mediators. Over the course of a two-hour assay in the presence of zVAD-fmk, ROS production decreased despite the loss of cytochrome c (**Figures 3-3B** and **3-8A**). With the loss of cytochrome c from mitochondria, uncoupling of electron transport and oxidative phosphorylation would be expected to occur leading ROS production at least in the long term. In our system however, unlike that of MacDonald *et al.*, substantial cytochrome c remains in mitochondria even at two hours (**Figure 3-8A**, lanes 5 and 12) and such cytochrome c levels may be sufficient to prevent complete uncoupling of electron transport and oxidative phosphorylation. It has recently been pointed out that the level of cytochrome c is not rate limiting for cellular respiration and that a fraction may be lost to the cytosol without adverse consequences to mitochondrial function (Crompton, 2000) Other potential sources of ROS production could be the ER and the cytoplasm (Pahl and Baeuerle, 1997) and these sources could potentially be coupled to caspase-dependent events.

In summary, these results provide evidence for a two-pronged mechanism for the destruction of target cells by CTL. The possibility of such a mechanism was first revealed by Sarin *et al.* (Sarin et al., 1997), and we now suggest that the caspase-independent pathway may be mediated through mitochondrial dysfunction. As depicted in **Figure 3-10**, once inside the cell, granzyme B can initiate apoptosis by the cleavage and activation of caspase-3. In addition, we demonstrate that the protease can also mediate caspase-independent effects on mitochondria that result in cytochrome c release and loss of $\Delta\Psi_m$. The release of cytochrome c will likely activate the Apaf-1/caspase-9 pathway but our data indicate that initial caspase-3 activation is achieved through direct cleavage by granzyme B resulting in apoptosis (Atkinson et al., 1998). In parallel with caspase-3 processing cells with dysfunctional mitochondria are also destined to die by necrosis. Such a two-pronged approach in vivo may serve to amplify

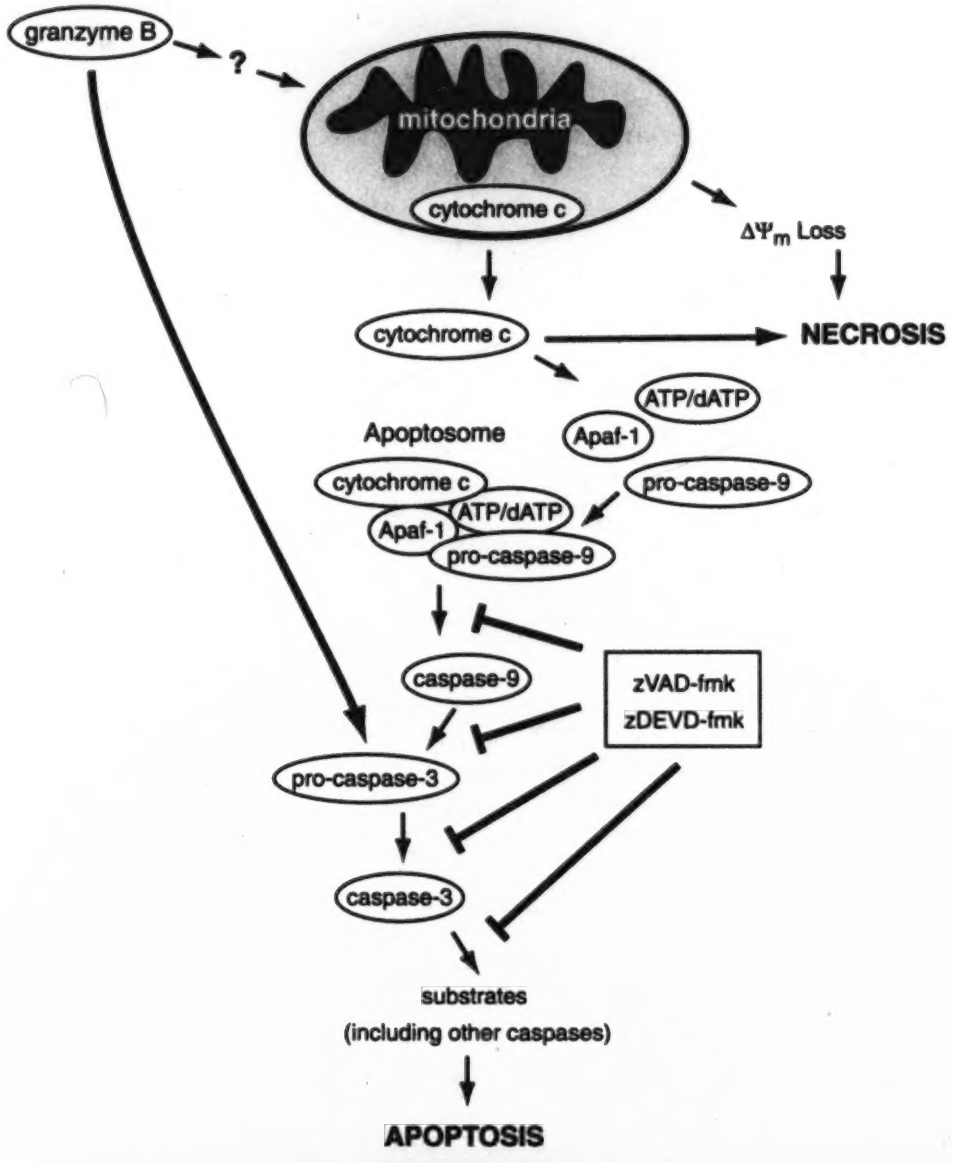


Figure 3-10 Model of granzyme B-mediated mitochondrial effects.

Granzyme B induces caspase-3 activation directly. However, granzyme B can also stimulate the loss of cytochrome c from mitochondria and a decrease in the $\Delta\Psi_m$ in a caspase-independent fashion. The loss of cytochrome c likely leads to an amplification of caspase-3 activation through an apoptosome-dependent process. In the presence of the caspase inhibitors zVAD-fmk or zDEVD-fmk, or in the absence of caspase-3, granzyme B-mediated loss of cytochrome c and/or $\Delta\Psi_m$ loss leads to cell death by necrosis.

caspase activation. Importantly, both the necrotic and apoptotic pathways are activated in response to granzyme B, clearly indicating that both pathways will have to be considered in therapeutic manipulations of the immune system.

Chapter 4

The Role of Bid and Bax in Granzyme B-Mediated Killing[1]

[1] A version of this chapter has previously been published: Jeffrey A. Heibein, Ing Swie Goping, Michele Barry, Michael J. Pinkoski, Gordon C. Shore, Douglas R. Green, and R. Chris Bleackley (2000) Granzyme B-Mediated Cytochrome c Release is Regulated by the Bcl-2 Family Members Bid and Bax. *J. Exp. Med.*, **192**, 1391-1401. *Copyright 2000. The Rockefeller University Press.*

Chapter 4: The Role of Bid and Bax in Granzyme B-Mediated Killing

4.1 Introduction

The destruction of target cells by CTLs is believed to involve either engagement of the Fas receptor or granule exocytosis. Fas ligation and oligomerization lead to the formation of the death inducing signaling complex (DISC) which begins with recruitment of FADD/MORT1 to the cytoplasmic death domains of Fas (Chinnaiyan et al., 1995). FADD acts as an adapter molecule between the Fas receptor and the pro-domain of caspase-8 (Boldin et al., 1996; Muzio et al., 1996). The recruitment of caspase-8 to the DISC brings identical molecules into close proximity and allows for catalytic activation. In cells where caspase-8 is efficiently recruited to the DISC, activated caspase-8 directly acts on caspase-3 (Scaffidi et al., 1998) to initiate the effector stages of the apoptotic program including chromatin condensation, DNA fragmentation, membrane alterations and cytoskeletal rearrangements (Enari et al., 1998; Kothakota et al., 1997; Porter and Jänicke, 1999; Stroh and Schulze-Osthoff, 1998; Wolfe et al., 1999). However in cells such as Jurkat, where recruitment of capsase-8 to the DISC is relatively inefficient, indirect activation of caspase-3 mediated through a mitochondrial pathway takes precedence. This occurs through proteolytic activation of Bid, a pro-apoptotic member of the Bcl-2 family (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). Caspase-8 cleavage of 26 kDa human Bid following Asp59 generates a C-terminal cleavage product of 15 kDa (Li et al., 1998; Luo et al., 1998; Wang et al., 1996). tBid translocates to mitochondria where it causes the permeablization of the outer mitochondrial membrane through a poorly defined mechanism leading to the release of cytochrome c from the intermembrane space (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). Once released to the cytoplasm,

cytochrome c acts as a cofactor in conjunction with Apaf-1, pro-caspase-9 and ATP/dATP to induce activation of caspase-9 and subsequently caspase-3 (Li et al., 1997; Zou et al., 1997).

The second mechanism utilized by CTL to kill targets involves the calcium dependent exocytosis of CTL-derived granule proteins and their uptake by the target cell. Among the granule components are the pore-forming protein perforin and a number of granule proteinases or granzymes, which gain entry into the target cell through a poorly defined mechanism (for review see Atkinson and Bleackley, 1995; Darmon et al., 1999; Kägi et al., 1996; Shresta et al., 1998). Granzyme B, the prototypic member of the granzymes, is a serine proteinase with a unique specificity among mammalian serine proteinases for cleavage on the carboxyl side of aspartate residues (Poe et al., 1991). A number of caspases contain cleavage sites potentially recognized by granzyme B (Harris et al., 1998; Vande Craen et al., 1997) implying that granzyme B may activate caspases in the target cell following uptake. Indeed caspases, including caspase-3 (CPP32/Apopain/Yama), caspase-6 (Mch2), caspase-7 (Mch3/CMH-1/ICE-LAP3), caspase-8 (FLICE/MACH/Mch5), capsase-9 (ICE-LAP6/Mch6) and caspase-10 (Mch4), are cleaved by granzyme B in vitro (Chinnaiyan et al., 1996; Darmon et al., 1995; Duan et al., 1996; Fernandes-Alnemri et al., 1996; Gu et al., 1996; Martin et al., 1996; Muzio et al., 1996; Orth et al., 1996; Quan et al., 1996; Srinivasula et al., 1996). In addition, processed caspases-1 (ICE), -3, -7, and -8 are found in whole cells following treatment with purified granzyme B and perforin or a replication-deficient adenovirus, or in response to treatment with human CTL (hCTL) (Atkinson et al., 1998; Barry et al., 2000; Chinnaiyan et al., 1996; Darmon et al., 1996; Shi et al., 1996).

Granzyme B can proteolytically cleave caspase-3 both in vitro and in whole cells in the presence of the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-

Ala-Asp-fluoromethyl ketone (zVAD-fmk) (Atkinson et al., 1998; Darmon et al., 1996; Darmon et al., 1995). This implies that granzyme B directly activates caspase-3 leading to apoptosis. In addition, it was recently shown that treatment of targets with granzyme B and adenovirus or perforin, or whole CTL leads to mitochondrial disruptions through a caspase-independent mechanism (Chapter 3 and MacDonald et al., 1999) implying that granzyme B can also induce necrotic cell death without caspase activation. However, it was not known whether granzyme B acted directly on mitochondria or indirectly through the proteolytic activation or inactivation of a cellular substrate(s).

We show here that Bid is required for the release of cytochrome c in response to granzyme B. Bid is known to be cleaved by granzyme B to generate a 14 kDa granzyme B-truncated Bid fragment (herein designated gtBid) that is distinct from tBid (Barry et al., 2000; Li et al., 1998). Here we present evidence that, once generated, gtBid translocates to mitochondria where it recruits the pro-apoptotic Bcl-2 family member, Bax, to the mitochondrial membrane. Bax becomes integrated into the membrane to induce release of cytochrome c through a caspase-independent mechanism. Importantly, Bcl-2 does not inhibit Bid cleavage and translocation to mitochondria but does prevent Bax insertion and cytochrome c release following treatment with granzyme B.

4.2 Results

4.2.1 A Cytosolic Factor is Required for Granzyme B-Mediated Cytochrome c Release.

It was previously shown that granzyme B can induce the loss of inner mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome c efflux from mitochondria through a caspase-independent mechanism (**Chapter 3**). To investigate whether granzyme B acted alone or in concert with additional factors we developed an in vitro

system that utilized purified mitochondria from Jurkat cells. Jurkat mitochondria were incubated either with granzyme B alone or Jurkat cytosol and granzyme B in various combinations for 30 minutes at room temperature either in the presence or absence of the broad-spectrum caspase inhibitor, zVAD-fmk. Following this incubation, the mixture was centrifuged and the mitochondrial pellets were separated from the supernatants. Proteins in the separated samples were resolved by electrophoresis and examined for the presence of cytochrome c by immunoblotting (**Figure 4-1**).

In the absence of treatment, cytochrome c was detectable only in the mitochondrial pellet and not in the supernatant (**Figure 4-1A**, lane 1 and compare lanes 12 to 2). When cytosol (**Figure 4-1A**, lanes 13 and 3) or granzyme B (**Figure 4-1A**, lanes 14 and 4) was added separately to mitochondria, cytochrome c release was not detected. However, when granzyme B was added to mitochondria in conjunction with cytosol, cytochrome c efflux from mitochondria was observed (**Figure 4-1A**, compare lanes 15 and 5). Importantly, the same results were observed in the presence of 100 μM zVAD-fmk (**Figure 4-1A**, compare lanes 33 and 24). Therefore, cytochrome c release did not occur through a direct interaction of granzyme B with mitochondria. Rather, a zVAD-fmk-insensitive cytosolic factor (or factors) was required.

4.2.2 Bid is Required for Granzyme B-Mediated Cytochrome c Release.

One possible candidate for the cytosolic factor required by granzyme B to elicit cytochrome c release was the pro-apoptotic Bcl-2 family member Bid. As outlined in the introduction, Bid has been shown to play a role in apoptosis induced after ligation of the Fas and TNF receptors (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). In addition to the caspase cleavage site at Asp59, Bid contains an excellent cleavage site for granzyme B ($IEAD^{75}$). We, and others, have recently shown that in the presence of

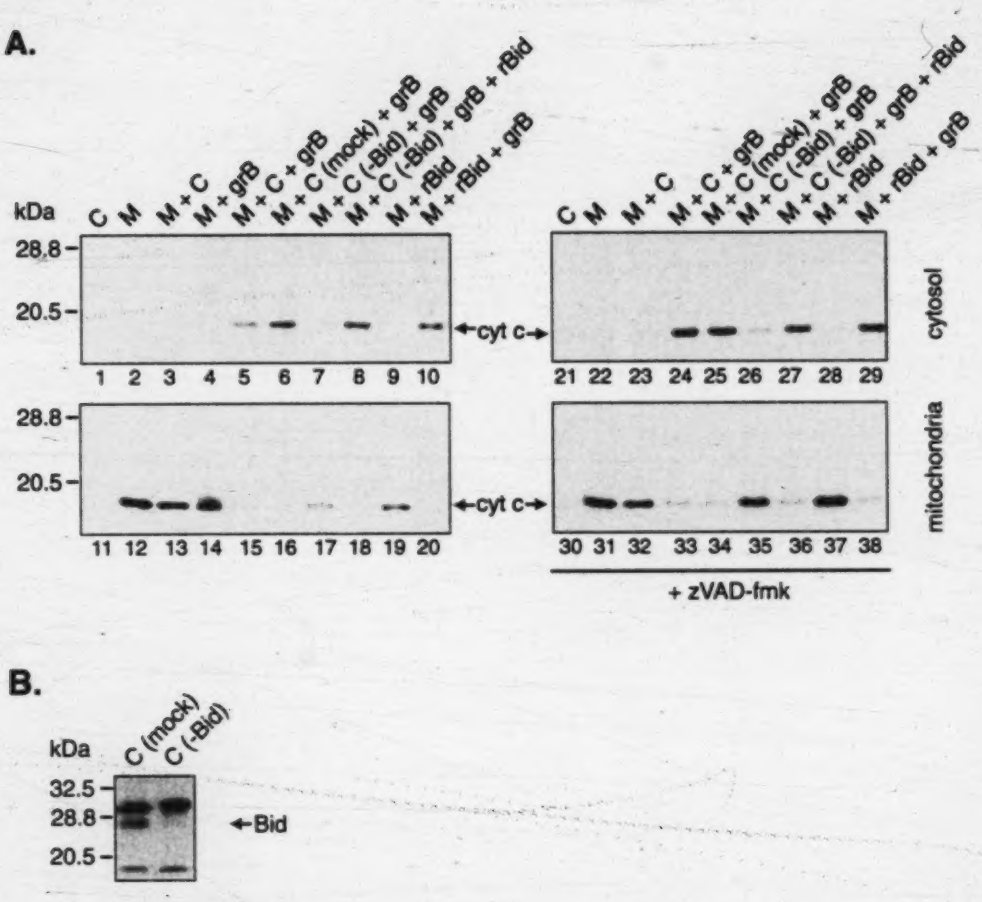


Figure 4-1 Granzyme B-mediated cytochrome c release from isolated mitochondria requires Bid.

Purified Jurkat mitochondria were treated with granzyme B and/or Jurkat cytosol for 30 minutes at room temperature in the presence or absence of 100 μM zVAD-fmk. The mitochondria/cytosol mixture was then centrifuged and the pellets (mitochondria) and supernatants (cytosol) resolved on 15% polyacrylamide gels followed by transfer to nitrocellulose and immunoblotting for cytochrome c. M, mitochondria; C, cytosol; grB, granzyme B; C(mock), mock immunodepleted cytosol; C(-Bid), cytosol immunodepleted for Bid; rBid, recombinant Bid. These data are representative of three independent experiments.

granzyme B, in vitro transcribed-translated Bid is cut into a 14 kDa product (Barry et al., 2000; Li et al., 1998) at Asp75 (Michael Pinkoski, personal communication). Moreover, Bid cleavage occurs in whole cells after treatment with granzyme B and adenovirus (Barry et al., 2000).

To determine if granzyme B-mediated cytochrome *c* efflux was dependent upon Bid, we immunodepleted Bid from the cytosolic extract prior to its addition to mitochondria plus granzyme B. The efficiency of Bid immunodepletion was assessed by western blotting (**Figure 4-1B**). Mock-immunodepleted cytosol generated the same cytochrome c efflux pattern as non-immunodepleted cytosol (**Figure 4-1A**, compare lanes 6 and 16 with lanes 15 and 5). In contrast, when the Bid-depleted cytosol was added to mitochondria in the presence of granzyme B, no release of cytochrome c was observed (**Figure 4-1A**, compare lanes 7 and 17). When recombinant Bid (rBid) was added back to the immunodepleted cytosol, cytochrome c efflux was restored but only upon the addition of granzyme B (**Figure 4-1A**, lanes 8 and 18). These data suggested that the loss of cytochrome c from mitochondria in response to granzyme B treatment was dependent on the presence of cytosolic Bid.

To confirm the dependence of cytochrome c release on Bid, mitochondria were treated with rBid in the absence of cytosol and in the presence or absence of granzyme B. Without granzyme B, rBid was unable to induce cytochrome c release from mitochondria (**Figure 4-1A**, lanes 9 and 19) Importantly, in contrast to rBid alone, treatment of mitochondria with rBid in conjunction with granzyme B led to the complete loss of cytochrome c (**Figure 4-1A**, lanes 10 and 20). These results imply that granzyme B-dependent proteolytic cleavage of Bid leads to the generation of a fragment that is active in mediating effects on mitochondria.

These experiments were repeated in the presence of zVAD-fmk with identical results (**Figure 4-1A**, compare lanes 21-29 to 30-38). Thus the granzyme B-mediated

cytochrome c efflux in this system occurred independently of caspases, consistent with the caspase-independent cytochrome c loss in whole cells seen following treatment with granzyme B and adenovirus (**Chapter 3, Figure 3-8**). Collectively, these data show that Bid is necessary for granzyme B-mediated cytochrome c release from mitochondria, after direct activation by this proteinase.

4.2.3 Granzyme B-Mediated Target Cell Death Induces Translocation of gtBid From the Cytosol to Mitochondria.

In response to ligation of Fas or TNF receptors, Bid is cleaved by caspase-8 to generate 15 kDa tBid. Translocation of tBid from the cytosol to mitochondria has been shown to be critical for cytochrome c release to occur (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). We wished to know if the smaller, granzyme-cleaved gtBid fragment also translocated to mitochondria in whole cells in response to granzyme B and adenovirus.

Jurkats were treated either with soluble anti-Fas antibodies or with granzyme B and adenovirus for the times indicated in the presence or absence of zVAD-fmk. Following treatment, cells were rendered permeable with digitonin and the mitochondrial fraction separated from the cytosol as described in **Chapter 2 (section 2.3.4)**. The proteins from these fractions were examined for Bid by immunoblotting (**Figure 4-2A**). We first confirmed the translocation of tBid in response to anti-Fas treatment (**Figure 4-2A**, lanes 1-12). In the absence of zVAD-fmk, cytosolic Bid was cleaved into 15 kDa tBid which was detectable in the mitochondrial fraction within 2-4 hours following anti-Fas addition (**Figure 4-2A** lanes 5 and 6). After longer incubation times, a greater quantity of tBid was seen in the mitochondrial fraction (compare lanes 5 and 6 of **Figure 4-2A**). In the presence of zVAD-fmk, the generation of tBid in response to Fas receptor ligation and its accumulation in mitochondria was not

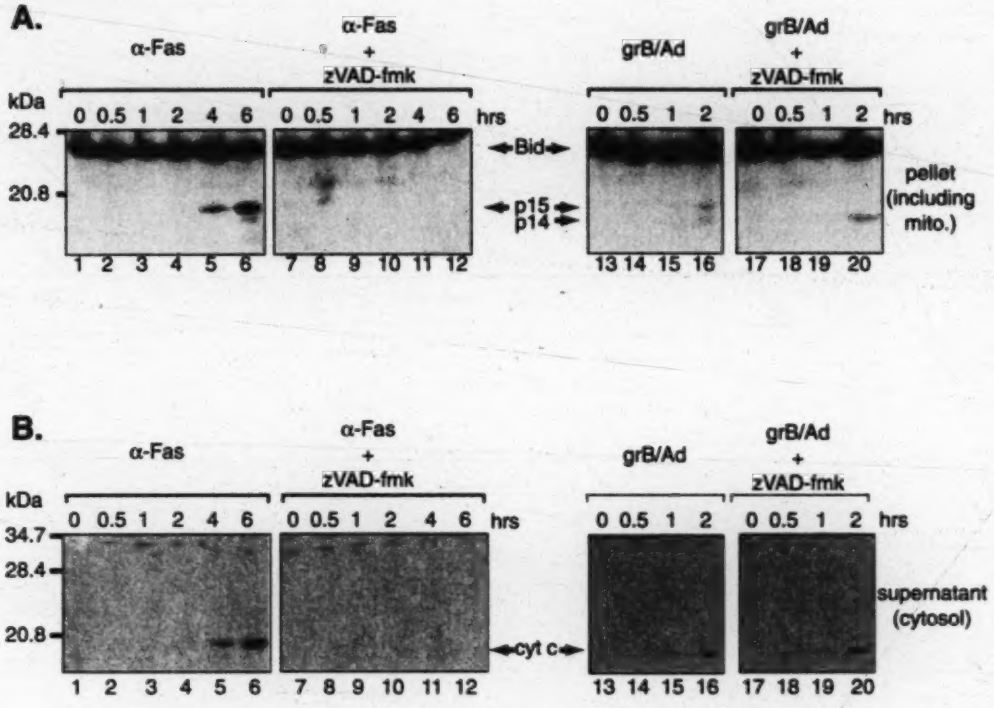


Figure 4-2 Cytosolic Bid is cleaved and translocates to mitochondria in response to granzyme B and adenovirus.

A. Jurkat targets were treated with either anti-Fas antibodies (lanes 1-12) or granzyme B and adenovirus (lanes 13-20) in the presence or absence of 100 μM zVAD-fmk. Following incubation at 37°C for the times indicated the plasma membranes of cells were rendered permeable with digitonin. The membrane fraction (containing mitochondria) was then separated from the cytosol by centrifugation. The pellets (mitochondrial fraction) were resolved on 15% polyacrylamide gels followed by transfer to nitrocellulose and immunoblotting for Bid using a rabbit polyclonal anti-Bid anti-serum. **B.** The supernatant blots from **A.** were stripped and re-probed for cytochrome c using a monoclonal anti-cytochrome c antibody. These data are representative of three independent experiments.

observed. These data are consistent with those previously published (Li et al., 1998; Luo et al., 1998).

In response to treatment with granzyme B and adenovirus, 14 kDa gtBid was generated that was detectable in the mitochondrial fraction within one hour following treatment (**Figure 4-2A**, lanes 15 and 16). In addition to 14 kDa gtBid, 15 kDa tBid was also generated which translocated to mitochondria (**Figure 4-2A**, lane 16). However, in the presence of zVAD-fmk only 14 kDa gtBid was seen in the mitochondria (**Figure 4-2A**, lane 20) indicating that the 15 kDa band was likely caspase-8-generated tBid.

The blots shown in **Figure 4-2A** were stripped and re-probed for cytochrome c as a control for mitochondrial dysfunction (**Figure 4-2B**). After anti-Fas treatment, cytochrome c release was observed (**Figure 4-2B**, lanes 1-6), Detectable cytochrome c release corresponding with the appearance of detectable tBid at the mitochondria (compare lanes 4-6 of **Figure 4-2A** with lanes 4-6 of **Figure 4-2B**). In the presence of zVAD-fmk, no cytochrome c release was observed following anti-Fas treatment (compare lanes 1-6 and 7-12 of **Figure 4-2B**) consistent with the caspase dependence of Fas-mediated cytochrome c release in Jurkats. Similarly, granzyme B-dependent loss of cytochrome c from mitochondria was observed (**Figure 4-2B**, lane 16) at the same time as gtBid was detected associated with mitochondria (compare lanes 13-20 of **Figure 4-2A** with lanes 13-20 of **Figure 4-2B**). However, unlike anti-Fas treatment, cytochrome c efflux occurred with similar kinetics in the presence of zVAD-fmk after treatment with granzyme B and adenovirus (compare lanes 15-16 and 19-20 of **Figure 4-2B**). Thus in whole cells, Bid cleavage by granzyme B generated 14 kDa gtBid which translocated to mitochondria coincident with the efflux of cytochrome c.

4.2.4 Bax is Integrated into the Mitochondrial Membrane Following Granzyme B and Adenovirus Treatment

Like Bid, another pro-apoptotic Bcl-2 family member, Bax, has been implicated in cytochrome c release (Eskes et al., 1998; Jürgensmeier et al., 1998; Narita et al., 1998; Priault et al., 1999). In some cases, Bid activation leads to a translocation or conformational change in Bax and its subsequent insertion into the outer mitochondrial membrane (Antonsson et al., 2000; Goping et al., 1998; Gross et al., 1998; Nechushtan et al., 1999; Wolter et al., 1997). To investigate whether Bax might be involved in granzyme B-mediated cytochrome c release, we utilized our whole cell killing system as indicated. Mitochondria were isolated from Jurkat targets following treatment with granzyme B and adenovirus and then subjected to alkaline extraction in 0.1 M Na_2CO_3 which strips away proteins peripherally associated with membranes while leaving integral membrane proteins intact (Goping et al., 1998). Bax was found in the cytosolic fraction and peripherally associated with mitochondria (data not shown) but not integrated into the mitochondrial membrane in control cells (**Figure 4-3**, compare lanes 1 and 6) or cells treated with either granzyme B alone (**Figure 4-3**, lanes 2 and 7) or adenovirus alone (**Figure 4-3**, lanes 3 and 8). However, following incubation with both granzyme B and Ad together, the levels of cytosolic Bax decreased (**Figure 4-3**, compare lanes 1 and 4) and Bax was found integrated into mitochondria (**Figure 4-3** lane 9). Loss of Bax from the cytosol and its appearance in the mitochondrial membrane was also observed in the presence of zVAD-fmk (**Figure 4-3**, lane 10). These data suggest that in response to treatment with granzyme B and adenovirus, Bax became integrated into the mitochondrial membranes of targets through a caspase-independent process.

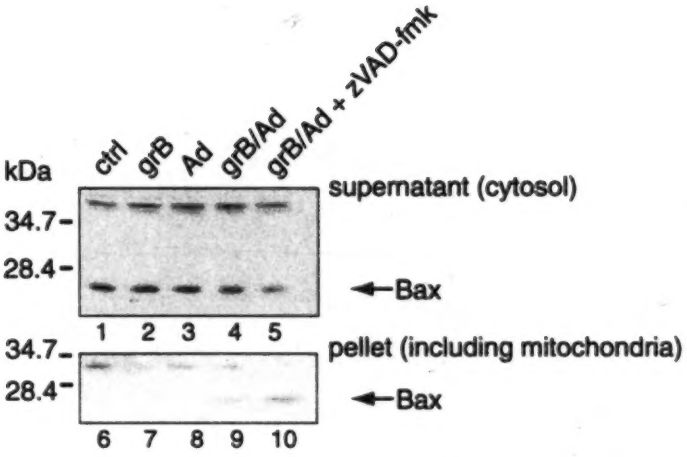


Figure 4-3 Bax is integrated into the mitochondrial membrane in response to granzyme B and adenovirus.

Jurkat targets were treated either with nothing (control), granzyme B alone, adenovirus alone or granzyme B and adenovirus (grB/Ad) in the presence or absence of 100 μM zVAD-fmk for 3 hours at 37°C. Following incubation, the plasma membranes of cells were rendered permeable with digitonin. The permeable cells were then centrifuged to separate the pellets (containing mitochondria) from the supernatants (cytosol). Pellets were subjected to an alkaline extraction with 0.1 M Na_2CO_3 for 30 minutes on ice followed by centrifugation. The pellets and the pre-alkaline extraction supernatants were resolved on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose and the blots probed with polyclonal anti-Bax antibodies. These data are representative of three independent experiments.

4.2.5 Granzyme B-Mediated Integration of Bax into the Mitochondrial Membrane is Dependent Upon Bid.

Since both gtBid translocation to mitochondria and Bax integration into mitochondrial membranes occurred in response to granzyme B and adenovirus in whole cells (**Figures 4-2A** and **4-3**), we sought to elucidate the relationship between Bax and Bid in granzyme B-mediated cytochrome c release. To this end, we treated isolated Jurkat mitochondria under various conditions followed by alkaline extraction (**Figure 4-4**). Bax was not found integrated with either mitochondria alone or mitochondria treated with Jurkat cytosol (**Figure 4-4**, lanes 1 and 2). However, when mitochondria were exposed to cytosol in conjunction with granzyme B, Bax was integrated into the membrane as indicated by the increase in the intensity of Bax immunolabeling in alkali-treated mitochondria (**Figure 4-4**, lane 3). When mock immunodepleted cytosol was used in place of regular Jurkat cytosol, no difference in Bax insertion was observed (**Figure 4-4**, lane 4). In contrast, when Bid was immunodepleted from Jurkat cytosol and the cytosol subsequently added to mitochondria in combination with granzyme B, Bax insertion was abrogated (**Figure 4-4**, lane 5). Indeed, in the absence of any other cytosolic components, rBid in combination with granzyme B was able to induce Bax integration into the mitochondrial membrane (**Figure 4-4**, lane 7). Detectable levels of Bax are found peripherally associated with Jurkat mitochondria (data not shown) which provided a source of Bax in the absence of cytosol. Presumably, the requirement for Bax insertion was gtBid since Bax insertion did not occur with rBid in the absence of granzyme B (**Figure 4-4**, lane 6). We observed the same results in the presence of zVAD-fmk (**Figure 4-4**, lanes 8-12) which indicated that in the granzyme system, caspases are not required for Bax insertion. Granzyme B-mediated Bax integration into rat heart mitochondria was also dependent on Bid when HeLa cytosolic extracts were used (data not shown).

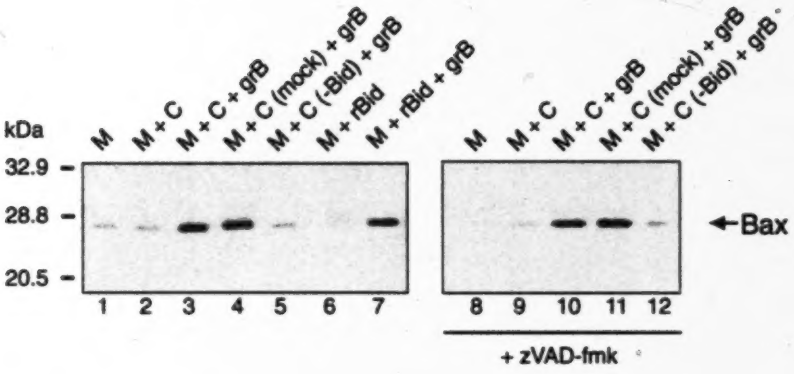


Figure 4-4 Granzyme B-mediated Bax integration into mitochondrial membranes requires Bid.

Purified Jurkat mitochondria (M) were treated with granzyme B and cytosol (C) for 30 minutes at room temperature in the presence or absence of 100 μM zVAD-fmk. Mitochondria were also treated with granzyme B in the presence of mock immunodepleted cytosol (C(mock)) and cytosol immunodepleted for Bid (C(-Bid)) as well as recombinant Bid (rBid). Following a 30 minute incubation at room temperature, the mitochondria were subjected to an alkaline extraction with 0.1 M Na_2CO_3 for 30 minutes on ice followed by centrifugation. The pellets were re-suspended in buffer A and resolved on 15% polyacrylamide gels followed by transfer to nitrocellulose and immunoblotting for Bax. These data are representative of three independent experiments. Data provided by Ing Swie Goping.

4.2.6 Bcl-2 Does Not Block Granzyme B-Mediated Bid Cleavage and Translocation but Does Prevent Bax Insertion and Cytochrome c Release

Both Bid and Bax have been implicated in cytochrome c release (Eskes et al., 1998; Gross et al., 1999; Jürgensmeier et al., 1998; Li et al., 1998; Luo et al., 1998; Narita et al., 1998; Priault et al., 1999). In our system, Bax insertion into mitochondria did not occur unless Bid was also present and processed by granzyme B (**Figure 4-4**). We wanted to determine if both Bid and Bax were required for granzyme B-mediated cytochrome c release and if so, to determine the order of recruitment of these proteins. The easiest method to investigate this would have been to immunodeplete Bax from cytosolic extracts and examine this effect on mitochondria in the presence of granzyme B. However, Jurkat mitochondria have significant levels of peripherally associated Bax following isolation (data not shown) precluding such an experimental approach. Nevertheless, Bcl-2 over-expression has been shown to block Bax translocation to mitochondria and subsequent cytochrome c release (Desagher et al., 1999; Eskes et al., 2000; Murphy et al., 2000; Murphy et al., 2000). We therefore examined the effect of granzyme B on Bid and Bax in Jurkat cells over-expressing Bcl-2 and in vector only transfectants (**Figure 4-5**).

Cells were manipulated as described for **Figure 4-2**. As with parental Jurkats (**Figure 4-2B**), cytochrome c release occurred in the vector only transfectants (neo) which was detectable within one hour following treatment with granzyme B and adenovirus (**Figure 4-5A**, lanes 1-4). However, cytochrome c was not detected in supernatants of the Bcl-2 transfectants under identical conditions (**Figure 4-5A**, lanes 5-8). Therefore, as has been previously published for a variety of apoptotic stimuli (Kluck et al., 1997; MacDonald et al., 1999; Yang et al., 1997), Bcl-2 blocked cytochrome c release in our system.

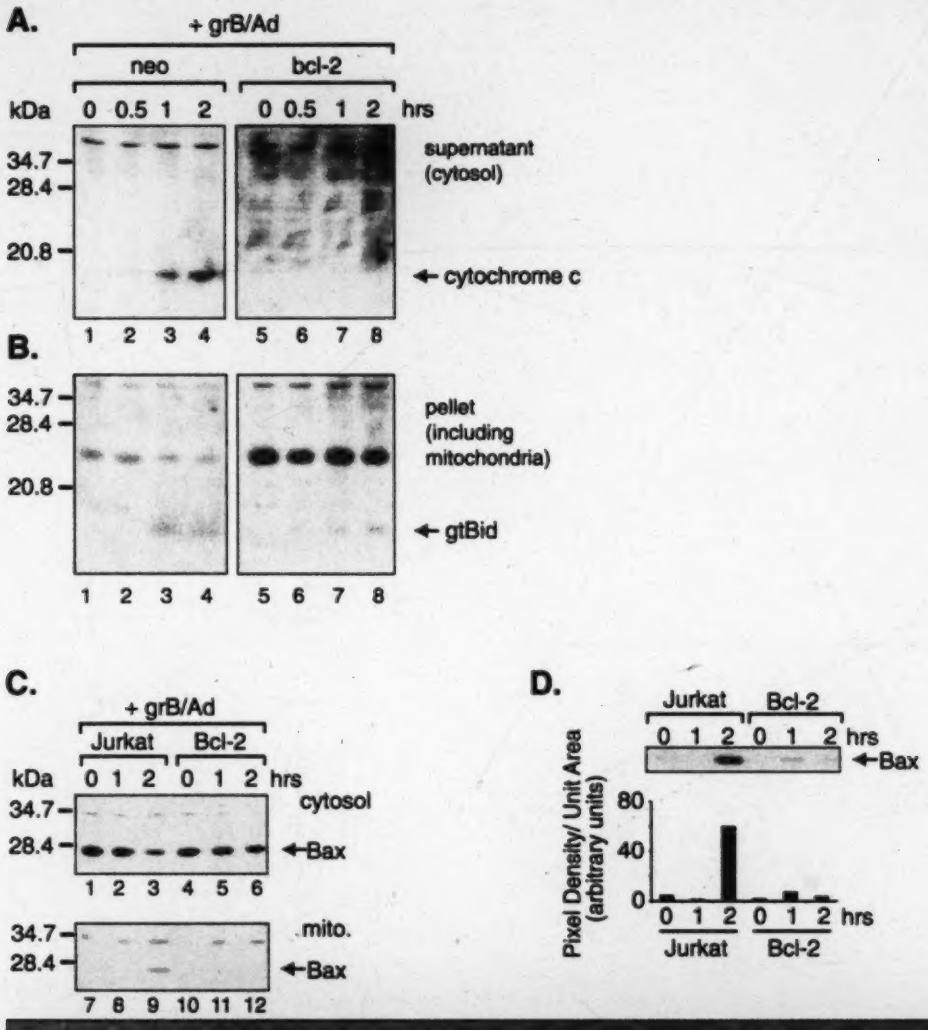


Figure 4-5 Bcl-2 blocks granzyme B-mediated Bax insertion and cytochrome c release but not Bid processing and translocation.

A. Vector-only transfected controls (neo) and Bcl-2 over-expressing Jurkats were treated with grazyme B and adenovirus for the times indicated. Following incubation, cells were fractionated, the proteins resolved on 15% gels and transferred to nitrocellulose. Cytosols were probed with a monoclonal anti-cytochrome c antibody. **B.** The mitochondrial fraction from **A.** was probed for Bid using a polyclonal anti-Bid antiserum. **C.** Jurkat and Bcl-2 over-expressing Jurkats were treated with granzyme B and adenovirus as indicated followed by sub-fractionation. The mitochondria were subjected to alkaline extraction followed by SDS-PAGE on 15% gels and transfer to nitrocellulose. Blots were probed with polyclonal anti-Bax antibodies. **D.** Mitochondrial fractions from **C.** were over-exposed (inset), scanned and then analyzed with Image Guage Software to determine relative band intensities.

We next examined Bid cleavage and translocation. Similar to parental Jurkats (**Figure 4-2**), Bid was processed to gtBid in the neo clones and detectable gtBid was found associated with mitochondria within one hour following granzyme B and Ad treatment (**Figure 4-5B**, lanes 1-4). Importantly, gtBid was also found to be associated with mitochondria in the Bcl-2 over-expressers with identical kinetics to the vector-only controls (**Figure 4-5B**, lanes 5-8).

Finally, we investigated the effect of Bcl-2 over-expression on Bax integration into the mitochondrial membrane (**Figure 4-5C**). For these experiments, cells were manipulated as described for **Figure 4-3**. The ideal control for these experiments would have been to compare the vector-only (neo) transfectants with the Bcl-2 over-expressers as for Bid and cytochrome c above (**Figure 4-5A** and **B**). However, our neo clones displayed unusually low levels of Bax relative to the parental Jurkat line (data not shown). Therefore, we compared the effects on Bax integration in the Bcl-2 over-expressers to the parental Jurkat line. Bax integration in Jurkat cells was detectable within two hours following granzyme B and adenovirus treatment (**Figure 4-5C**, lanes 1-3). However, no Bax incorporation was observed in the Bcl-2 over-expressing cells (**Figure 4-5C**, lanes 4-6). A slight difference in the quantity of cytosolic Bax between parental Jurkat and Bcl-2 transfectants was seen when the blots were scanned and the intensity of the bands measured. At time zero, the intensity of the Bax band of the Bcl-2 over-expressers was ~13 % lower than that of the parental Jurkat line (data not shown). The fact that there was slightly less cytosolic Bax in the Bcl-2 transfectants might suggest that the absence of Bax in the mitochondrial fraction simply resulted from less starting material. However, when the blots containing the mitochondrial fractions were over-exposed overnight (**Figure 4-5D**, inset) and the intensity of the bands corresponding to Bax was measured (**Figure 4-5D**), a nearly 15-fold increase in intensity of Bax was seen in the parental Jurkat mitochondria after two hours. In

contrast, no more than a three-fold increase was seen with the Bcl-2 transfectants. If Bax integration into mitochondria occurred despite the presence of Bcl-2, a ~13% decrease in the starting levels of Bax would still allow it to be detected in mitochondria, particularly with longer exposures. However, virtually no Bax was detected even at longer exposures. Collectively, these data indicated that the over-expression of Bcl-2 blocked granzyme B-mediated Bax integration into mitochondria and cytochrome c release, but did not affect Bid processing and translocation.

4.3 Discussion

The induction of apoptosis in a pathogenic cell under attack by a CTL is known to involve granzyme B activation of caspases, notably caspase-3. However, this paradigm does not explain the caspase-independent arm of CTL-mediated death. Our previous experiments demonstrated that mitochondrial changes might be an important component of the death machinery activated by CTL (**Chapter 3**). Since cytochrome c efflux in response to granzyme B occurred in a caspase-independent fashion, granzyme B was either acting directly on mitochondria to induce cytochrome c release, or indirectly through a cellular substrate which was not a caspase. Data presented here indicates that granzyme B alone is unable to elicit the release of cytochrome c from purified mitochondria (**Figures 4-1** and **4-2**). Rather, the pro-apoptotic Bcl-2 family members Bid and Bax are required in conjunction with granzyme B.

We examined whether Bid was required for cytochrome c efflux in an in vitro system using purified Jurkat mitochondria and purified Jurkat cytosol. Immunodepletion of Bid from the cytosol abolished, while re-introduction of recombinant Bid (rBid) to the Bid-depleted cytosol restored the ability of granzyme B to induce cytochrome c efflux. These data are consistent with those already published indicating that the immunodepletion of Bid from TNF/cycloheximide-treated cytosolic extracts prevented cytochrome c release (Gross et al., 1999). However in our system, granzyme B had to

be present in conjunction with cytosol suggesting that either Bid is processed directly or through other granzyme-activated proteinases.

In the absence of cytosol, the addition of rBid to mitochondria was an insufficient stimulus for cytochrome c release. However, cytochrome c efflux was accomplished by adding granzyme B and rBid to mitochondria indicating that caspases were clearly not required to mediate this effect. These data contrast with the process of Bid cleavage characterized for the Fas pathway that depends upon caspase-8 to process Bid (Li et al., 1998; Luo et al., 1998).

In response to ligation of the Fas receptor, caspase-8 cleavage of Bid at Asp59 generates a 15 kDa tBid C-terminal fragment. Removal of the N-terminus of Bid exposes the otherwise inaccessible BH3 domain found in the C-terminus of full-length Bid (Chou et al., 1999; McDonnell et al., 1999). Once formed, tBid rapidly translocates to mitochondria to induce cytochrome c release through an ill-defined mechanism (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). Processing of Bid by granzyme B at Asp75 generates a 14 kDa gtBid product (Li et al., 1998) that, like tBid, contains the BH3 domain. Despite the different site of cleavage and the smaller size of the cleavage product, gtBid was found localized to mitochondria in whole cells in response to treatment with granzyme B and adenovirus. Since the BH1, BH2 and BH3 domains of Bcl-2/Bcl-X_L can act as a binding pocket for the BH3 domains of other Bcl-2 family members (Sattler et al., 1997; Wang et al., 1996), freeing the BH3 domain of Bid may allow it to heterodimerize with Bcl-2/Bcl-X_L on the mitochondrial membrane. In support of this notion, one study reported that recombinant tBid had a 10-fold higher affinity for GST-Bcl-X_L than did full-length Bid (Li et al., 1998). In addition, mutations in Bid's BH3 domain that prevented its binding to Bcl-2/Bcl-X_L also blocked cytochrome c release and apoptosis (Gross et al., 1999; Luo et al., 1998; Wang et al., 1996). However, these BH3 mutations did not block translocation of Bid to mitochondria. In addition to

exposing the BH3 domain, the removal of the N-terminus of Bid increases the surface hydrophobicity of tBid and alters its surface charge (Chou et al., 1999; McDonnell et al., 1999). It is these changes, rather than exposure of the BH3 domain, which may allow tBid to target membranes. Nevertheless, by whatever mechanism tBid associates with mitochondria, the regions of tBid that are required for this process are clearly also present in gtBid.

In the absence of zVAD-fmk, tBid was also generated in response to granzyme B and adenovirus treatment (Barry et al., 2000; Medema et al., 1997). Although not required for apoptosis, granzyme B can cleave and activate caspase-8 (Barry et al., 2000). Since tBid is generated by caspase-8, it remained possible that despite the presence of gtBid, cytochrome c release resulted from tBid translocation alone. However, we showed here that in the presence of zVAD-fmk, cytochrome c release occurred but only gtBid was detected. Importantly, detectable cytochrome c release paralleled detectable gtBid translocation either in the absence or presence of zVAD-fmk. This indicated that the smaller gtBid was equivalent to the larger caspase-8-generated tBid in its ability to induce cytochrome c efflux from mitochondria. Therefore, our in vitro and in vivo data demonstrates for the first time that caspase-independent cytochrome c release is dependent on granzyme B-mediated cleavage of Bid and the subsequent translocation to mitochondria of gtBid.

The mechanism by which Bid induces cytochrome c release remains elusive. Nevertheless, mounting evidence suggests that, at least in some cases, the re-localization of tBid functions to recruit another pro-apoptotic Bcl-2 family member, Bax, to mitochondria (Desagher et al., 1999). Bax recruitment may occur through a tBid-induced conformational change in Bax that allows it to insert into the mitochondrial outer membrane (Eskes et al., 2000). In Jurkat cells following treatment with granzyme B and adenovirus, Bax was found to integrate into mitochondrial membranes in both

the presence and absence of zVAD-fmk. These results are consistent with data showing that in the presence of recombinant tBid, Bax translocation to mitochondria and its integration into the outer membrane does not rely upon caspases (Desagher et al., 1999; Eskes et al., 2000; Murphy et al., 2000). Here we show that Bax integration in response to granzyme B also occurred with purified mitochondria in a caspase-independent fashion but only when Bid was present in cytosolic extracts. Thus, as previously described for tBid, gtBid was able to recruit Bax to mitochondria.

What role Bax may play in gtBid-induced cytochrome c release is unclear. Bax itself has been shown to induce cytochrome c release in isolated mitochondria (Eskes et al., 1998; Jürgensmeier et al., 1998; Narita et al., 1998; Priault et al., 1999) and when over-expressed in whole cells (Pastorino et al., 1998). Recently, Bax was shown to interact with the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane to form a cytochrome c conducting channel (Narita et al., 1998; Shimizu et al., 1999; Shimizu and Tsujimoto, 2000). Bax may also interact with the adenine nucleotide transporter (ANT) on the inner mitochondrial membrane to cause a permeability transition leading to an indirect release of cytochrome c (Marzo et al., 1998). Cyclosporine, an inhibitor of the permeability transition, blocked this Bax-mediated cytochrome c release (Marzo et al., 1998). However, other evidence indicates that Bax-mediated cytochrome c release is cyclosporine-insensitive and therefore independent of the permeability transition (Eskes et al., 2000). This argues that the manner in which Bax affects cytochrome c release may be differentially regulated in different cells and/or in response to different apoptotic stimuli. In the granzyme system, both $\Delta\Psi_m$ loss and cell death seen in whole cells is cyclosporine-independent (Chapter 3 and MacDonald et al., 1999), arguing that the recruitment of Bax to mitochondria and its role in cytochrome c release are independent of the permeability transition.

Bcl-2 has been reported to block cytochrome c release in response to a variety of apoptotic stimuli (Kluck et al., 1997; Yang et al., 1997). Further, Bcl-2 blocks both translocation of Bax from the cytosol to mitochondria and its subsequent insertion into the outer membrane (Desagher et al., 1999; Eskes et al., 2000; Murphy et al., 2000; Murphy et al., 2000). In response to granzyme B and adenovirus treatment, no cytochrome c release was seen in Jurkat cells over-expressing Bcl-2. However, even with Bcl-2 over-expression, granzyme B-dependent Bid cleavage still occurred and gtBid was found re-localized to mitochondria. Despite gtBid translocation to mitochondria in the presence of Bcl-2, no Bax integration into the mitochondrial membrane was observed. The Bcl-2-antagonized integration of Bax correlated with the absence of cytochrome c release in response to granzyme B and adenovirus. Thus, while gtBid was localized to mitochondria in the Bcl-2 over-expressing cells, no cytochrome c release was observed in the absence of Bax integration. We cannot rule out the possibility that the levels or functions of other Bcl-2 family members may be altered in the Bcl-2 transfectants and that the effects of these proteins may influence the movement of Bax to mitochondria. Nevertheless, there is now growing evidence in the literature to link Bid with Bax recruitment to mitochondria (Desagher et al., 1999; Eskes et al., 2000; Ruffolo et al., 2000). In addition, the literature supports a model whereby Bax recruitment can be antagonized by Bcl-2 (Murphy et al., 2000; Murphy et al., 1999; Murphy et al., 2000). Finally, evidence suggests that Bax is able to induce the efflux of cytochrome c from mitochondria (Antonsson et al., 2000; Desagher et al., 1999; Finucane et al., 1999; Jürgensmeier et al., 1998; Narita et al., 1998; Priault et al., 1999). We have not shown that cytochrome c release absolutely depends upon Bax integration into the mitochondrial outer membrane in the granzyme system. However, the fact that cytochrome c release does not occur in the absence of Bax integration coupled with the available literature renders this hypothesis plausible.

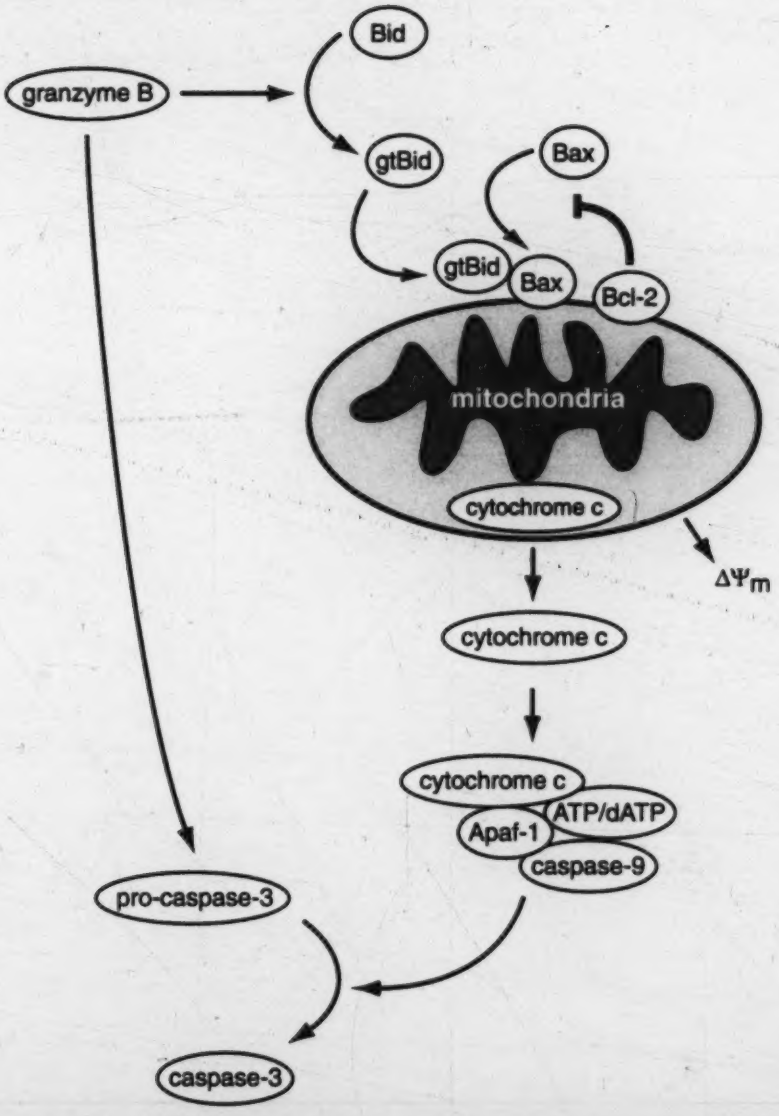


Figure 4-6 Model for regulation by Bcl-2 family members of granzyme B-mediated cytochrome c release.

Granzyme B may activate caspase-3 directly, or indirectly through the proteolytic activation of Bid. Bid is cleaved to generate gtBid that translocates to mitochondria where it recruits Bax through a mechanism antagonized by Bcl-2. Bax becomes integrated into the mitochondrial membrane and induces cytochrome c release. Cytochrome c acts as a cofactor with Apaf-1, caspase-9 and ATP/dATP to foster the proteolytic activation of caspase-3.

Collectively our data supports a model in which granzyme B proteolytically cleaves Bid to generate a 14 kDa gtBid fragment that translocates to mitochondria through a caspase-independent mechanism (**Figure 4-6**). gtBid localized to mitochondria then recruits Bax to the mitochondrial outer membrane through a mechanism antagonized by Bcl-2. Once integrated, Bax initiates cytochrome c release from mitochondria through a cyclosporine-independent mechanism.

It has been suggested that caspase inhibitors may be effective in the treatment of autoimmune disorders and suppression of transplant rejection. Our results imply that these alone would be ineffective. Rather, therapeutic interventions that block both caspase and mitochondrial pathways would be required. The relative importance of these mechanisms in cell death in vivo is currently under investigation.

Chapter 5

Caspases Accelerate Granzyme B-Mediated Killing

Chapter 5: Caspases Accelerate CTL-Mediated Killing

5.1 Introduction

It is generally believed that apoptosis induction by granzyme B results from the direct proteolytic activation of pro-caspase-3. Unquestionably, there is experimental evidence to support such a model. Caspase-3 is cleaved by granzyme B in vitro (Darmon et al., 1996; Darmon et al., 1995; Martin et al., 1996; Quan et al., 1996) and processed caspase-3 is found in targets treated with granzyme B and perforin (Chinnaiyan et al., 1996) or whole CTL (Atkinson et al., 1998; Darmon et al., 1996). In these targets, the 32 kDa pro-caspase-3 is cleaved to generate a 20 kDa C-terminal fragment (p20) that is subsequently autocatalytically cleaved at Asp9. This cleavage removes the short pro-domain thereby generating a 19 kDa active caspase-3 enzyme (Atkinson et al., 1998). Addition of the inhibitor of caspase-3-like caspases, zDEVD-fmk, or the broad-spectrum inhibitor, zVAD-fmk, to targets inhibits apoptosis mediated by allogeneic CTL (Atkinson et al., 1998). However, these inhibitors do not prevent the initial cleavage event of pro-caspase-3 to the p20 fragment consistent with direct cleavage by granzyme B (Atkinson et al., 1998).

Granzyme B-dependent activation of caspase-3 is clearly required for a number of morphological features of target cell apoptosis. Pharmacological inhibitors of caspases, such as zVAD-fmk or zDEVD-fmk, block DNA fragmentation, phosphatidylserine externalization and ROS production (**Chapter 3**). However, in the presence of caspase-inhibition, $\Delta\Psi_m$ loss still occurs as does cytochrome c release and ultimately the cells die, albeit through a more 'necrosis-like' process (**Chapter 3**). Hence the data presented in **Chapter 3** as well as work from other labs support the existence of caspase-independent pathways utilized by CTL to kill targets (MacDonald et al., 1999; Sarin et al., 1998; Sarin et al., 1997).

Despite the fact that granzyme B can kill cells in the absence of caspase activation, the reality in a physiological setting is that granzyme B-mediated killing leads to the activation of caspases (Atkinson et al., 1998; Barry et al., 2000; Chinnaiyan et al., 1996; Darmon et al., 1996; Medema et al., 1997; Shi et al., 1996). Caspases have been shown to enhance death responses through caspase cascades in which active caspases cleave and activate additional caspases (Cohen, 1997) (also see **Figure 1.6.2**). In addition, $\Delta\Psi_m$ loss has also been attributed to caspases (Castedo et al., 1996; Green and Kroemer, 1998; Susin et al., 1997) and caspase-8 can process Bid, leading to the translocation of tBid to mitochondria and subsequently cytochrome c release (Li et al., 1998; Luo et al., 1998). Thus, in a physiological setting, caspases may have effects on killing resulting from their contributions to feed-forward pathways involving mitochondria. Indeed, the role of mitochondria as accelerators of granzyme B-mediated killing has recently been described (MacDonald et al., 1999).

With these functions of caspases in mind, it was of interest to further examine the involvement of these enzymes in CTL-mediated killing in both our in vitro system using purified granzyme B and adenovirus and in our in vivo system using hCTL. It was found that while caspase inhibition with the broad-spectrum caspase inhibitor, zVAD-fmk, ultimately provided no abrogation of $\Delta\Psi_m$ loss at later time points consistent with previous findings (**Chapter 3**), it did delay the onset of $\Delta\Psi_m$ loss in both our in vitro and in vivo systems. Inhibition of caspase-3-like caspases with zDEVD-fmk provided no such effect suggesting that effector caspases do not substantially participate in the mitochondrial effects observed in targets. In addition, caspase-3 processing by granzyme B was delayed in these targets although cleavage to p20 was observed. These data are consistent with the involvement of initiator caspases in accelerating granzyme B-mediated cell death.

5.2 Results

5.2.1 Caspase Inhibitors Block Early but not Late Granzyme B-Mediated $\Delta\Psi_m$ Loss

The inhibition of caspases with either the broad-spectrum caspase inhibitor zVAD-fmk or with the inhibitor of caspase-3-like caspases, zDEVD-fmk clearly does not impede the ultimate disruption of mitochondrial dysfunction in cells treated with granzyme B and adenovirus or granzyme B and perforin (**Chapter 3**). Nevertheless, much evidence in the literature supports a role for both mitochondria and caspases in feed-forward mechanisms leading to the acceleration of death signals culminating in cellular demise (Green and Reed, 1998; Nicholson, 1999). While it is evident that granzyme B-mediated killing can proceed in the absence of caspase activation (**Chapter 3**), in a physiological setting it is probable that both caspase-dependent and caspase-independent pathways would be activated. Therefore, time course studies were undertaken to investigate the relative contribution of caspases in mitochondrial dysfunction mediated by granzyme B and adenovirus.

The effect of caspase-inhibitors on mitochondrial dysfunction was first examined in an in vitro system. JpEGFP targets were used for the in vitro experiments as a control since these were the targets used for the whole cell killing assays described in the next section. Targets were pre-loaded with DASPEI and then treated with granzyme B and adenovirus for up to three hours in the presence or absence of zVAD-fmk or zDEVD-fmk. The cells were then examined for loss of $\Delta\Psi_m$ as measured by loss of DASPEI from targets as described for **Chapter 3**. Following the addition of granzyme B and adenovirus, $\Delta\Psi_m$ dysregulation was observed while in the absence of treatment (control), no loss of $\Delta\Psi_m$ was seen (**Figure 5-1 A** and **B**). In the presence of either zVAD-fmk or zDEVD-fmk, $\Delta\Psi_m$ loss was observed by three hours, consistent with the end-point data presented in **Chapter 3**, **Figure 3-3**. Importantly, however, at

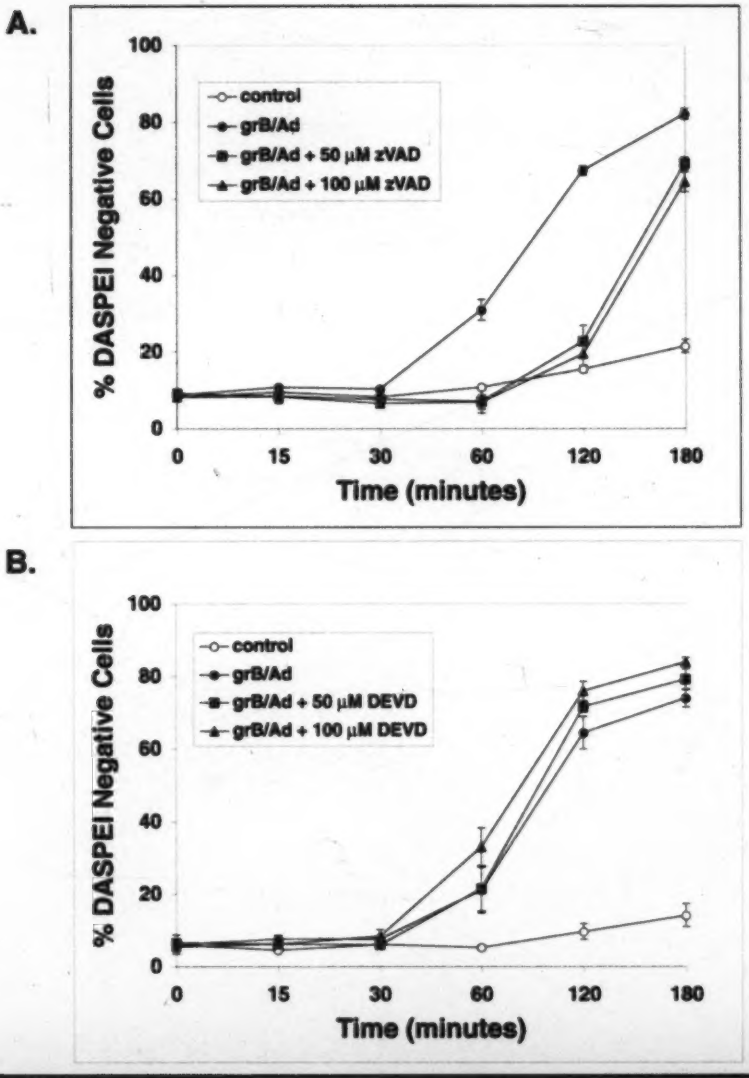


Figure 5-1 Caspase inhibition differentially affects $\Delta\Psi_m$ loss in response to treatment with granzyme B and adenovirus.

GFP-expressing Jurkat targets (JpEGFP) were pre-loaded with 50 mM DASPEI and then incubated at 37°C with granzyme B (1 µg/ml) and adenovirus (MOI= 10 pfu/cell) for the times indicated. **A.** Incubation with zVAD-fmk causes a delay in $\Delta\Psi_m$ loss. Studies were performed in the absence (closed circles) or the presence of zVAD-fmk (closed squares: 50 µM zVAD; closed triangles: 100 µM zVAD). Control cells (open circle) recieved no treatment. **B.** Incubation with zDEVD-fmk has little effect on $\Delta\Psi_m$. Studies were performed in the absence (closed circles) or the presence of zDEVD-fmk (closed squares: 50 µM zDEVD; closed triangles: 100 µM zDEVD). Control cells (open circle) recieved no treatment. Means and standard deviations of three expriements shown.

either 50 or 100 μM zVAD-fmk, $\Delta\Psi_m$ disruption was clearly delayed even up to two hours following treatment with granzyme B and adenovirus (**Figure 5-1A**). This discrepancy in mitochondrial dysfunction was essentially non-existent by three hours.

Interestingly, treatment with zDEVD-fmk, an inhibitor of caspase-3-like enzymes, yielded different results. Unlike the inhibition seen with the broad-spectrum inhibitor zVAD-fmk, inhibition of caspase-3-like caspases with zDEVD-fmk did not impart a significant kinetic delay in $\Delta\Psi_m$ disruption at early or late time points (**Figure 5-1B**). Collectively, these data indicate that inhibition of caspases other than caspase-3-like enzymes prevents the early $\Delta\Psi_m$ loss observed in response to granzyme B and adenovirus treatment. However, despite the caspase-dependent delay, virtually complete $\Delta\Psi_m$ loss was ultimately observed.

5.2.2 Caspase Inhibitors Block Early but not Late CTL-Mediated $\Delta\Psi_m$ Loss

We wished to know if the effects of caspase inhibition observed for granzyme B and adenovirus were also occurring in cells in response to treatment with whole CTL. To examine this, GFP-expressing Jurkats (JpEGFP) were pre-loaded with DASPEI and exposed to hCTL in the presence or absence of zVAD-fmk (**Figure 5-2**) or zDEVD-fmk (**Figure 5-3**) for up to three hours. At a 1:1 effector to target ratio, zVAD-fmk had a similar effect on $\Delta\Psi_m$ loss to that observed with granzyme B and adenovirus treatment (**Figure 5-2**). Disruption of $\Delta\Psi_m$ was blocked at early time points (~15-20% DASPEI negative cells in the presence of 50 or 100 μM zVAD-fmk versus ~60% in the absence of zVAD-fmk at one hour). However, by two hours following the addition of hCTL to targets, $\Delta\Psi_m$ loss was approaching that seen in the absence of zVAD-fmk (~30-40% DASPEI negative cells in the presence of 100 or 50 μM zVAD-fmk respectively versus ~75% in the absence of zVAD-fmk). By three hours, $\Delta\Psi_m$ loss in the zVAD-fmk-treated cells was only slightly lower than that observed in the absence of treatment (~60-70%

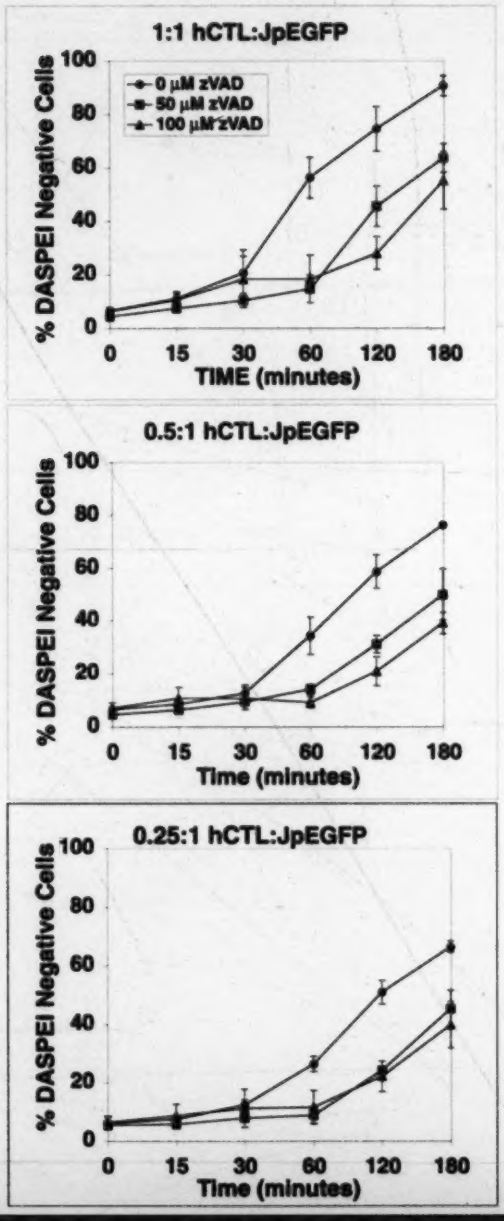


Figure 5-2 Caspase inhibition with zVAD-fmk delays $\Delta\Psi'_m$ loss in response to treatment with hCTL.

GFP-expressing Jurkat targets (JpEGFP) were pre-loaded with 50 mM DASPEI and then incubated at 37°C with hCTL at the effector to target ratios (hCTL:JpEGFP) indicated. Incubations were performed in the absence (closed circles) or the presence of zVAD-fmk (closed squares: 50 µM zVAD; closed triangles: 100 µM zVAD). Means and standard deviations of three experiments shown.

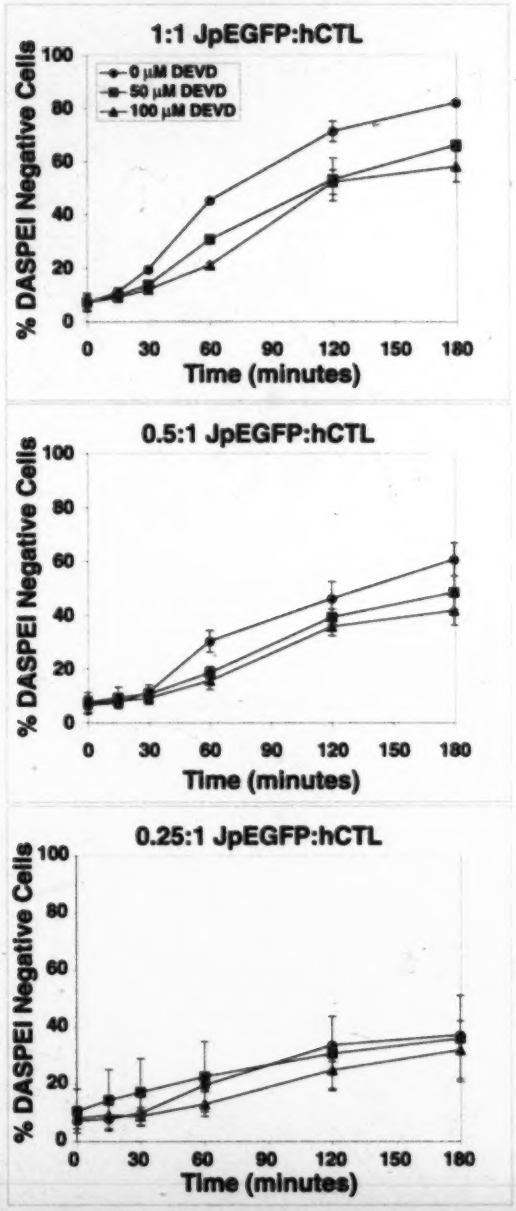


Figure 5-3 Caspase inhibition with zDEVD-fmk does not significantly affect $\Delta\Psi_m$ loss in response to treatment with hCTL.

GFP-expressing Jurkat targets (JpEGFP) were pre-loaded with 50 mM DASPEI followed by incubation at 37°C with hCTL at the effector to target ratios (hCTL:JpEGFP) indicated. Incubations were performed in the absence (closed circles) or the presence of zDEVD-fmk (closed squares: 50 μM zDEVD; closed triangles: 100 μM zDEVD). Means and standard deviations of two independent experiments shown.

for 50 and 100 μM zVAD-fmk respectively versus 90% in the absence of zVAD-fmk). The effect of zVAD-fmk on $\Delta\Psi_m$ loss was paralleled almost identically with lower effector to target ratios (0.5:1 and 0.25:1; **Figure 5-2**).

The experiments outlined for zVAD-fmk were also performed using zDEVD-fmk (**Figure 5-3**). The results from these experiments differed from those with zVAD-fmk in that there was no profound inhibition of $\Delta\Psi_m$ loss at any time during the course of the assays. At higher effector to target ratios (1:1), there was an approximately 10-15% decrease in the levels of $\Delta\Psi_m$ loss from 1 to 3 hours in the presence of zDEVD-fmk. However, these differences became less apparent as the effector to target ratios decreased (0.5:1 and 0.25:1). These data with hCTL mirror those observed for treatment of targets with granzyme B and adenovirus (**Figure 5-1**). zDEVD-fmk offered no significant protection against $\Delta\Psi_m$ loss at all time points examined. Importantly, unlike the data for zVAD-fmk, zDEVD-fmk did not cause a delay in the kinetics of $\Delta\Psi_m$ disruption again suggesting that caspase-3-like caspases were not responsible for the acceleration effects on mitochondria. Collectively, the inhibitor studies with whole CTL are consistent with the inhibitor studies in vitro where targets were treated with granzyme B and adenovirus (**Figure 5-1**).

5.2.3 Caspase Inhibition Retards Activation of Caspase-3 by Granzyme B

In Jurkat targets exposed to hCTL, caspase-3 is cleaved at Asp175 to generate a 20 kDa cleavage product (Atkinson et al., 1998). Caspase-3 is further processed through autocatalytic removal of the N-terminal pro-domain to generate a p19 fragment. A p17 fragment is also sometimes observed although the significance of this cleavage product is not clear. The processing of caspase-3 to the initial p20 fragment in response to hCTL is likely granzyme B-dependent since granzyme B is the only known mammalian serine proteinase with an aspartate specificity and since generation

of p20 occurs even in the presence of caspase inhibition by zVAD-fmk (Atkinson et al., 1998). However, we wished to know if caspase-3 was cleaved in Jurkat targets in response to granzyme B and adenovirus and if so, what effect caspase inhibition had on these events.

To determine the effects of zVAD-fmk on granzyme B-mediated caspase-3 activation, we treated Jurkat targets with granzyme B and adenovirus for up to two hours in the presence or absence of 100 μM zVAD-fmk (**Figure 5-4**). Following incubation, cells were separated into membrane and cytosolic fractions as outlined for the cytochrome c assays presented in **Chapter 3** (see **Chapter 2, section 2.3.4**). Cytosolic proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and the membranes immunoblotted for caspase-3. In the absence of zVAD-fmk, 32 kDa pro-caspase-3 was cleaved to generate p20 and p19 as early as 30 minutes following initiation of the assay (**Figure 5-4** lane 2). p20 remained a faint band relative to p19 and the intensity of p19 increased from 30 minutes to two hours (**Figure 5-4** lanes 2-4). By two hours, a p17 fragment was also apparent in the absence of caspase inhibition (**Figure 5-4** lane 4). In distinct contrast, zVAD-fmk-mediated caspase inhibition led to a complete absence of p19 and p17 products. The generation of p20 occurred within 30 minutes following the addition of granzyme B and adenovirus (**Figure 5-4** lane 6), however, the intensity of this band was weaker than the p19 band seen at the same time point in the absence of zVAD-fmk (**Figure 5-4** lane 2). Importantly, when examined up to two hours following the onset of treatment, p20 was the only cleavage product observed and the intensity of this band continued to increase (**Figure 5-4** lanes 6-8). Of note, however, the intensity of the p20 band by two hours in the presence of zVAD-fmk (**Figure 5-4** lane 8) was less than the cumulative intensities of the p20, p19 and p17 bands by two hours in the absence of zVAD-fmk (**Figure 5-4** lane 4). Collectively, however, these data support the notion that granzyme B can directly

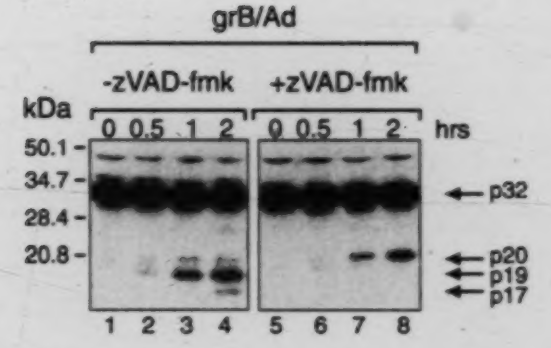


Figure 5-4 Caspase inhibition with zVAD-fmk does not prevent caspase-3 processing by granzyme B.

Jurkat targets were treated for the times indicated at 37°C with granzyme B (1 µg/ml) and adenovirus (MOI=10 pfu/cell) in the presence and absence of 100 µM zVAD-fmk. Following incubation, cells were separated into membrane and cytosolic fractions as outlined in **section 2.3.4** of **Chapter 2**. The proteins within the cytosolic fractions were then resolved by SDS-PAGE (15% gels) and subsequently transferred to nitrocellulose. Immunoblotting for caspase-3 was performed using affinity purified polyclonal anti-caspase-3 antibodies and ECL. Data representative of two independent experiments.

cleave caspase-3 at Asp175 to generate the p20 product but indicate a significant attenuation of capsase-3 cleavage by zVAD-fmk.

5.3 Discussion

The data of **Chapter 3** provided evidence that caspases are not required for cell death mediated through the granzyme B pathway. In the presence of caspase inhibition, DNA fragmentation, phosphatidylserine externalization, and ROS production do not occur, but both cytochrome c release and loss of $\Delta\Psi_m$ are observed and cells die by a process more reminiscent of necrosis (**Figures 3-3** and **3-8**). However, we now show that while $\Delta\Psi_m$ loss occurs in a caspase-independent fashion, $\Delta\Psi_m$ loss followed delayed kinetics in response to whole CTL and granzyme B and adenovirus in the presence of the broad-spectrum caspase-inhibitor zVAD-fmk (**Figures 5-1A** and **5-2**). How does one explain the kinetic differences in $\Delta\Psi_m$ loss seen with zVAD-fmk when ultimately, $\Delta\Psi_m$ loss occurs despite the absence of active caspases?

One possible explanation for the kinetic differences observed is that caspases, while clearly not essential for granzyme B-mediated cell death, act to accelerate granzyme B-induced mitochondrial events. One zVAD-fmk-sensitive caspase that has been implicated with mitochondrial disruption in the Fas pathway is caspase-8. Following oligomerization of the Fas receptor, caspase-8 is activated and cleaves Bid. The C-terminal truncation product (tBid) then translocates to mitochondria where it induces loss of cytochrome c and $\Delta\Psi_m$ through an ill-defined mechanism (Gross et al., 1999; Li et al., 1998; Luo et al., 1998). Although granzyme B-dependent killing clearly does not depend upon the direct or indirect activation of caspase-8 (Barry et al., 2000), caspase-8 is nevertheless processed in targets in response to treatment with granzyme B and adenovirus or whole CTL (Barry et al., 2000; Medema et al., 1997). Therefore, it is possible in vivo that efficient and rapid granzyme-B dependent

disruption of mitochondrial function involves both the activation of Bid by granzyme B (**Chapter 4**), in addition to the activation of caspases such as caspase-3 and caspase-8 that can also act on mitochondria.

In contrast to the effects of zVAD-fmk on $\Delta\Psi_m$ loss, only a slight kinetic alteration was seen in the presence of the inhibitor of caspase-3-like caspases, zDEVD-fmk (**Figures 5-1B** and **5-3**). This indicates a differential sensitivity of mitochondria to granzyme B-mediated caspase activation. It appears that caspase-3-like caspases are not required to increase the efficiency of $\Delta\Psi_m$ loss although we have shown that they are clearly required for DNA fragmentation (**Chapter 3, Figure 3-9**). The slight inhibition of $\Delta\Psi_m$ loss seen with zDEVD-fmk is, however, consistent with the proposed positive feedback role for caspase-3 in caspase-9 activation (Slee et al., 1999). In this model, caspase-3 is able to directly cleave pro-caspase-9 in the absence of apoptosome formation (Slee et al., 1999). However, the effects of caspase-3-like enzymes must, at least in vivo, be relatively minimal when compared to the contribution of other caspases to mitochondria-dependent acceleration of cell death. This is because the initial kinetic delay imparted by broad caspase inhibition is significantly more profound than that observed in the presence of inhibition of caspase-3 like caspases only. These data are consistent with the established roles of caspase-3-like caspases as effector caspases responsible for instigating the morphological features of apoptosis rather than the initiation events of death pathways (Earnshaw et al., 1999; Nicholson, 1999; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). However, it is important to recognize that while $\Delta\Psi_m$ loss may be delayed and DNA fragmentation blocked by caspase inhibition (**Figures 3-8, 4-1** and **4-2**), membrane damage and cell lysis ultimately occur and so inhibiting caspases does not prevent cell death. Rather, caspase inhibition serves to shift the morphology of cell death from apoptosis to necrosis and perhaps to retard the onset of this latter morphology.

Interestingly, while there appears to be a caspase-dependence to the kinetics of $\Delta\Psi_m$ loss, we have been unable to detect any differences in the kinetics of cytochrome c release in the presence of zVAD-fmk (**Chapter 3, Figure 3-8**). Following treatment with granzyme B and adenovirus, cytochrome c was detected in the cytoplasm of both Jurkat and HeLa targets within 30 minutes either in the absence or the presence of 100 μM zVAD-fmk (**Chapter 3, Figure 3-8**). Although caspases may be important for accelerating events leading to $\Delta\Psi_m$ disruption, the data of **Figure 3-8** imply that granzyme B activity alone is sufficient to induce loss of cytochrome c from mitochondria. Nevertheless, caspase-8 is processed by granzyme B in vivo (Barry et al., 2000; Medema et al., 1997) and caspase-8 can cleave Bid to elicit the efflux of cytochrome c from mitochondria (Li et al., 1998; Luo et al., 1998). It seems plausible then that caspase-8 could also accelerate cytochrome c release. However, as indicated, we have never observed a kinetic delay in cytochrome c release in the presence of zVAD-fmk. The data presented in **Chapter 4** suggests that in addition to granzyme B-dependent activation of caspase-8 subsequently leading to the cleavage of Bid, granzyme B can directly process Bid leading to cytochrome c release. Since nanogram quantities of granzyme-processed Bid are sufficient to elicit cytochrome c efflux, at least in vitro (Goping and Bleackley, unpublished observations), it is possible that the contribution of caspase-8-cleaved Bid to this process is negligible and therefore no alterations in the kinetics of cytochrome c release were seen.

We have previously reported that the processing of caspase-3 in targets following treatment with hCTL occurs through the direct proteolytic action of granzyme B on caspase-3. This conclusion was based upon data showing that the initial cleavage event at Asp175 to generate a 20 kDa fragment (p20) still occurred in the presence of zVAD-fmk (Atkinson et al., 1998). With hCTL, there appeared to be no alteration in the kinetics of caspase-3 processing to produce the p20 fragment. In the presence of

zVAD-fmk or in its absence, p20 appeared within 15 minutes following addition of hCTL to Jurkat targets. The data presented here with granzyme B and adenovirus are consistent with those published for hCTL in that granzyme B can directly cleave caspase-3 at Asp175. In contrast with whole CTLs, however, with granzyme B and adenovirus in the presence of zVAD-fmk, there was a slight delay in the kinetics of caspase-3 cleavage to the p20 (**Figure 5-4**). In addition, by the two-hour end-point of the assay, less p20 was generated than the total amounts of p20, p19 and p17 in the absence of caspase-inhibition (**Figure 5-4,** compare lanes 4 and 8). This supports direct processing of caspase-3 by granzyme B, but suggests that other zVAD-fmk-sensitive enzymes are likely to participate in caspase-3 activation in vivo.

Since granzyme B can cause cytochrome c release, one possible scenario is that cytochrome c allows apoptosome formation and caspase-9 activation and it is caspase-9 that mediates the zVAD-fmk-sensitive caspase-3 processing to generate p19 and p17. Additionally, however, caspase-8 has been shown to directly activate caspase-3 (Scaffidi et al., 1998; Stennicke et al., 1998) and so further caspase-3 processing could result from caspase-8-dependent activity. At the moment, we cannot distinguish between the involvement of one or the other of these caspases or for that matter, other zVAD-fmk-insensitive proteases. However, the availability of Jurkat targets transfected with the rabbitpox virus serpin, SPI-2, renders this question testable. SPI-2 is an excellent inhibitor of caspase-8 (Barry et al., 2000) and if caspase-3 processing to p19 or p17 occurs in the SPI-2-expressing cells, then caspase-8 cannot be the enzyme responsible for these events. The identity of the caspase or caspases contributing to caspase-3 processing is currently under investigation.

Collectively, the data presented in this chapter are consistent with a model whereby granzyme B induces the loss of cytochrome c and $\Delta\Psi_m$ through both caspase-dependent and caspase-independent mechanisms (**Figure 5-5**). In this model,

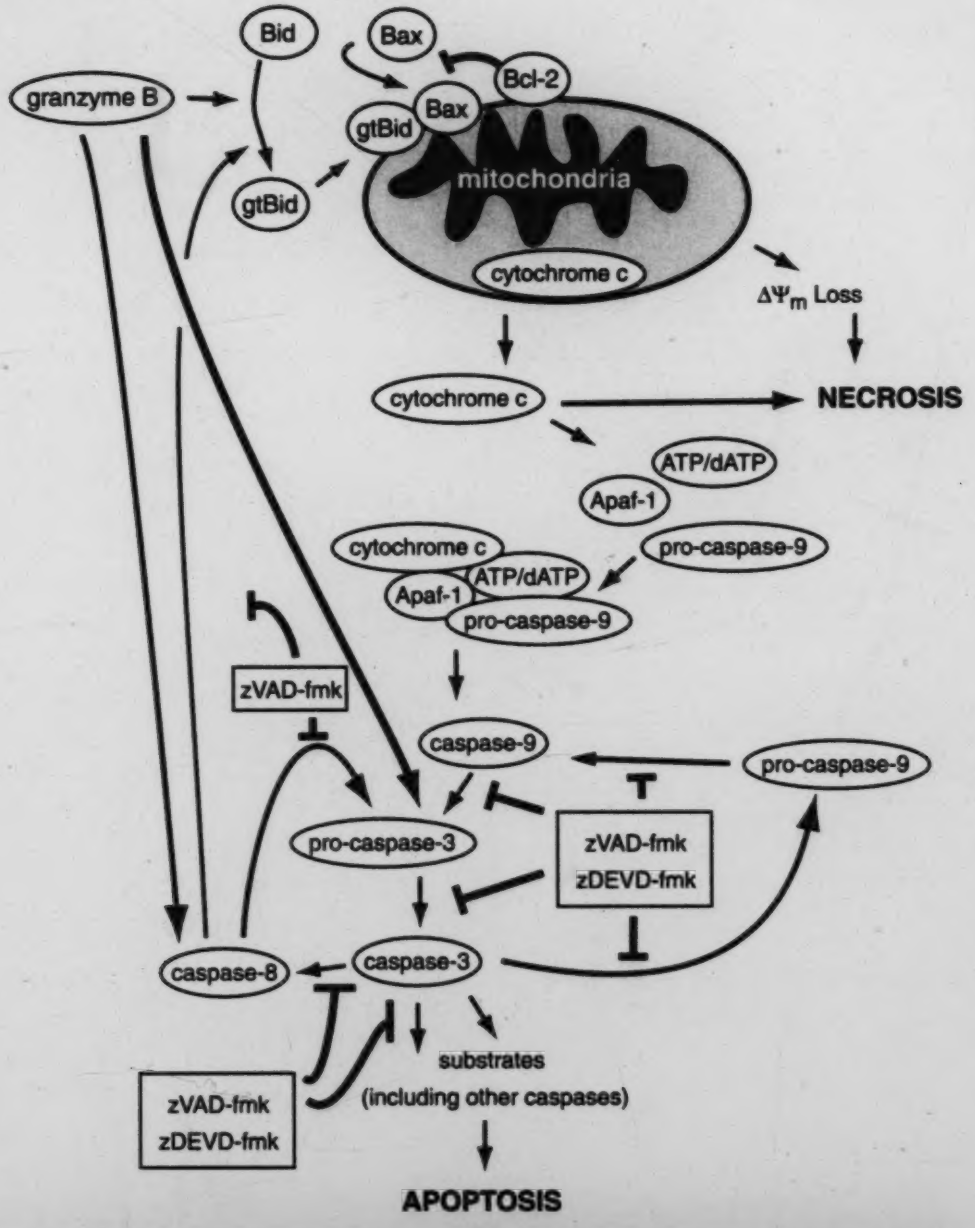


Figure 5-5 Model for the involvement of caspases in accelerating granzyme B-mediated cell death.

Granzyme B can process pro-caspase-3 either directly or indirectly through a mitochondrial pathway involving apoptosome formation. Once active, caspase-3 can process other caspases such as pro-caspase-9 and pro-caspase-8 that feedback onto mitochondria or mitochondrial pathways to accelerate the apoptotic response.

granzyme B cleaves Bid to gtBid that then recruits Bax to the mitochondrial membrane to elicit cytochrome c efflux through an unknown process antagonized by Bcl-2 (**Chapter 4**). Cytosolic cytochrome c then permits apoptosome formation and activation of caspase-9 leading to the activation of caspase-3. Alternatively, granzyme B can process caspase-3 directly. Active casapse-3 cleaves and inactivates or activates a number of cellular substrates including caspase-8 and caspase-9 that act to accelerate mitochondrial disruption. In the presence of the caspase inhibitors zVAD-fmk and zDEVD-fmk, granzyme B is still able to directly process caspase-3 and caspase-8, however, neither caspase is enzymatically active, thus abrogating the accelerating effects directed through mitochondria. Ultimately, in the absence of caspase activation and subsequent apoptotic morphologies, mitochondria lose cytochrome c, $\Delta\Psi_m$, and the cells will die by necrosis. However, the ability of CTLs to access apoptotic feed-forward mechanisms would be advantageous in situations, for example, where the rapid elimination of virus-infected cells could prevent the further spread of virus.

Chapter 6

Bcl-2 Retards Caspase-3 Cleavage by Granzyme B and Blocks the Appearance of Caspase-3 Enzymatic Activity

Chapter 6: Bcl-2 Retards Caspase-3 Cleavage by Granzyme B and Blocks the Appearance of Caspase-3 Enzymatic Activity

6.1 Introduction

It is now recognized that mitochondrial events including loss of $\Delta\Psi_m$ and cytochrome c release occur during granzyme B-mediated cell death (Chapter 3 and MacDonald et al., 1999). The involvement of mitochondria hints at a role in CTL-mediated cell death for the proto-oncogene product Bcl-2. Members of the Bcl-2 family of proteins are clearly involved in the regulation of apoptosis (for review see Chao and Korsmeyer, 1998; Korsmeyer et al., 1993; Kroemer, 1997). Bcl-2 is widely regarded as an anti-apoptotic molecule and much experimental evidence supports the ability of this protein to prevent apoptosis in response to a variety of apoptotic stimuli in myriad cell types. This inhibition likely occurs through the ability of Bcl-2 to block the release of mitochondrial cytochrome c and $\Delta\Psi_m$ loss (Kluck et al., 1997; Yang et al., 1997) thereby blocking activation of caspases downstream of mitochondria. Indeed, Bcl-2 was able to block Bax integration into the mitochondrial membrane and subsequent cytochrome c release in response to granzyme B and adenovirus (**Chapter 4**).

Despite the effects of Bcl-2 on cytochrome c release in our in vitro system, the effects of Bcl-2 on CTL-mediated killing remain controversial and confusing. In some systems, where mouse myeloid and lymphoid targets were pitted against allogeneic CTL, or purified NK cell granules, Bcl-2 offered no protection against killing as measured by cell lysis and DNA fragmentation (Sutton et al., 1997). However, Bcl-2 blocked apoptosis when purified granzyme B and perforin were used against these same targets (Sutton et al., 1997). In contrast, in other cell types killed exclusively through the granule pathway, and at low effector to target ratios, Bcl-2 blocked early

DNA fragmentation, although with longer incubations this inhibition was overcome (Renvoizé et al., 1997). Additionally, these studies also showed that despite impairing DNA fragmentation, Bcl-2 provided no protective effects against membrane damage, and cell death ultimately occurred (Renvoizé et al., 1997). Another study attributed similar membrane damage to the pore-forming effects of perforin (Torigoe et al., 1994). In still other systems, whole CTL that killed exclusively through the granule pathway or purified granules were unable to eliminate targets over-expressing Bcl-2 as measured either by DNA fragmentation or lytic damage (Chiu et al., 1995). Some of these differences could be explained by the involvement of mitochondrial pathways by some target cell types but not others. This differential recruitment of pathways in different cell types in response to a single stimulus has been described for killing following ligation of the Fas receptor (Scaffidi et al., 1998). In response to Fas receptor ligation in some cells (designated type I), caspase-3 activation occurs by the direct proteolytic action of caspase-8. In contrast, in type II cells, caspase-3 activation depends upon caspase-8 cleaving the pro-apoptotic family member Bid to trigger a mitochondrial pathway to capsase-3 activation (Scaffidi et al., 1998). Since the mitochondrial pathway can be antagonized by Bcl-2, type II cells show resistance to CTL-mediated killing whereas type I cells do not.

Mitochondrial pathways are activated in our killing system in response to granzyme B and adenovirus (**Chapter 3** and **Chapter 4**). In addition, Jurkat cells have been designated as type II since in response to Fas receptor ligation, Jurkats die predominantly through activation of the mitochondrial pathway (Scaffidi et al., 1998). Moreover, Bcl-2 blocked gtBid-induced Bax integration and subsequent cytochrome c release. Therefore, we wished to know if Bcl-2 could regulate additional aspects of granzyme B-dependent killing of Jurkat targets.

Our results show that when Bcl-2-expressing targets were treated with purified granzyme B and adenovirus, apoptosis was blocked as measured by the absence of DNA fragmentation, phosphatidylserine externalization, $\Delta\Psi_m$ loss, reactive oxygen species (ROS) production and cytochrome c release consistent with the activation by granzyme B of the mitochondrial pathway. In addition, caspase-3 processing by granzyme B was delayed in these targets although cleavage to p20 was observed. Hence, Bcl-2 can block granzyme B-dependent killing but does not affect the direct processing of caspase-3 by granzyme B. Interestingly, however, while caspase-3 was processed, no enzymatic activity was detected indicating that Bcl-2 affects the regulation of multiple caspases downstream of mitochondria. Importantly, in contrast to the inhibitory effects of Bcl-2 on granzyme B-mediated killing, Bcl-2 was unable to prevent apoptosis in targets exposed to whole CTL. This suggests that the granules contain other proteins capable of inducing target cell apoptosis.

6.2 Results

6.2.1 Bcl-2 Blocks Apoptosis in Response to Granzyme B Treatment

As indicated in **section 6.1**, the role of Bcl-2 in CTL-mediated killing remains polemical. However, since Bcl-2 was clearly influencing cytochrome c release in response to gtBid, it was of interest to further examine the role of Bcl-2 in granzyme B-dependent killing. We first examined the effects of Bcl-2 over-expression in Jurkats on apoptosis induced by granzyme B and adenovirus or granzyme B and perforin (**Figure 6-1**). Two clones (clones 3 and 5) showing similar levels of Bcl-2 expression (data not shown) as well as a vector-only control (BMGneo) showing levels of Bcl-2 expression identical to that of the parental Jurkat line (data not shown) were chosen for these experiments. The parental Jurkats, vector-only control (BMGneo) and the Bcl-2 clones were treated with granzyme B and adenovirus or granzyme B and perforin for 2 hours

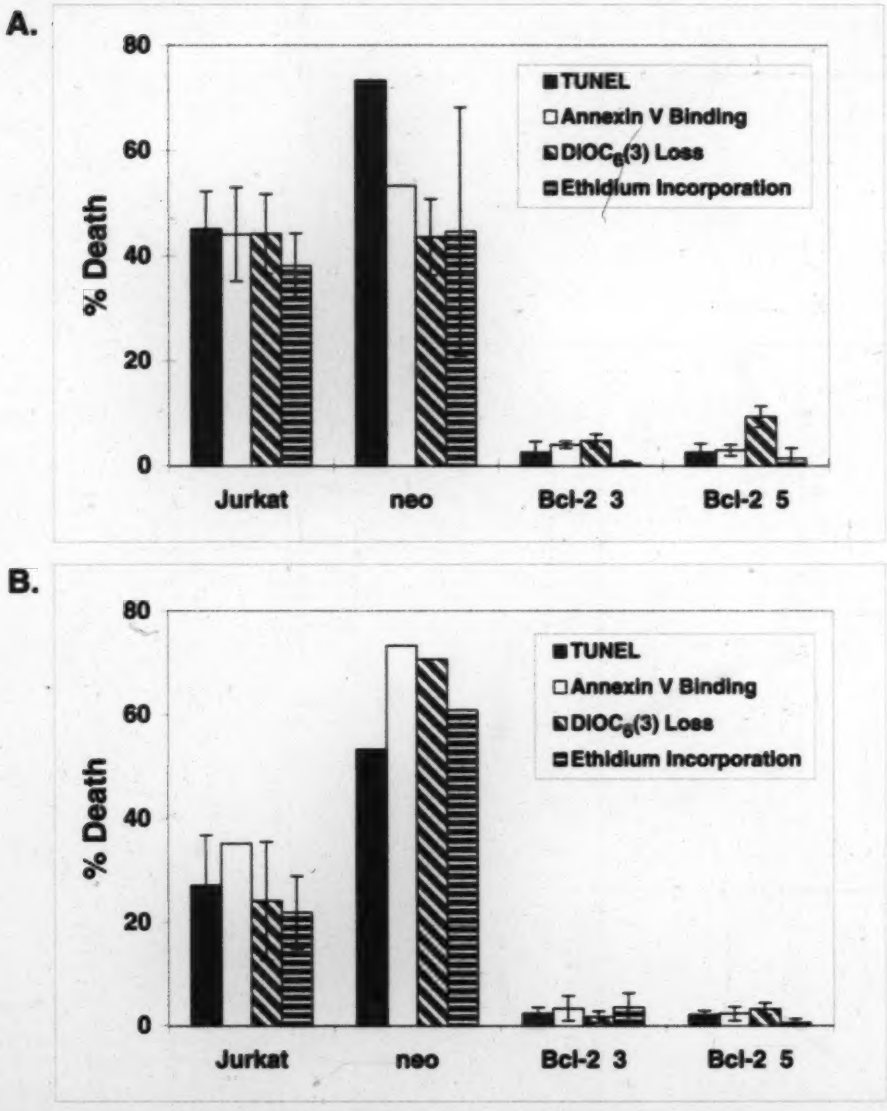


Figure 6-1 Bcl-2 blocks apoptosis mediated by granzyme B and adenovirus or granzyme B and perforin.

A. Targets (either parental Jurkats, vector-only controls (neo), or Jurkats over-epressing Bcl-2 (clones 3 and 5)) were incubated with 1 μg/ml granzyme B and adenovirus (MOI=10) for two hours at 37°C. Apoptosis was determined by examining DNA fragmentation (TUNEL), phosphatidylserine externalization (annexin V binding), $\Delta\Psi_m$ ($DiOC_6(3)$ loss) and ROS production (ethidium incorporation) as outlined in **Chapter 2 (section 2.3)**. **B.** Identical to **A.** but cells were treated with granzyme B and perforin.

at 37°C followed by assays for apoptosis. The features of apoptosis examined included DNA fragmentation (TUNEL), phosphatidylserine externalization (annexin V binding), $\Delta\Psi_m$ damage ($DiOC_6(3)$ loss) and ROS production (ethidium incorporation). When either granzyme B and adenovirus or granzyme B and perforin were utilized, the results were indistinguishable. The parental Jurkat targets were seen to undergo apoptosis by all measures (**Figure 6-1**). Similarly, although to a somewhat greater extent, the BMGneo controls also underwent apoptosis by all measures. Importantly, however, when the Bcl-2 over-expressing clones were investigated, DNA fragmentation, phosphatidylserine externalization, $\Delta\Psi_m$ loss and ROS production was nearly completely abrogated (**Figure 6-1**).

In addition to the downstream events of apoptosis, Bcl-2 has been shown to block cytochrome c release in response to a number of apoptotic stimuli (Kluck et al., 1997; Yang et al., 1997). Since mitochondrial effects clearly occur in the granzyme system (**Chapter 3**) and since Bcl-2 appeared to prevent $\Delta\Psi_m$ disruption in vitro (**Figure 6-1**), we wished to know what effect Bcl-2 had on granzyme B-mediated cytochrome c release. Bcl-2 over-expressing clones and the BMGneo (neo) vector-only control cells were treated with granzyme B and adenovirus for up to four hours followed by analysis for the loss of cytochrome c from mitochondria to the cytosol (**Figure 6-2**). As seen with parental Jurkats (**Chapter 3, Figure 3-8**), treatment of the neo targets with granzyme B and adenovirus led to the appearance of cytochrome c in the cytoplasm. Cytochrome c release was apparent by two hours in this assay (**Figure 6-2** lane 2) and by four hours, the level of cytochrome c in the cytoplasm had increased (**Figure 6-2** lane 3). In contrast, treatment of Bcl-2 over-expressing Jurkat targets prevented the loss of mitochondrial cytochrome c even at four hours post-treatment (**Figure 6-2** lanes 4-6). Collectively, the data presented in this section shows that in our

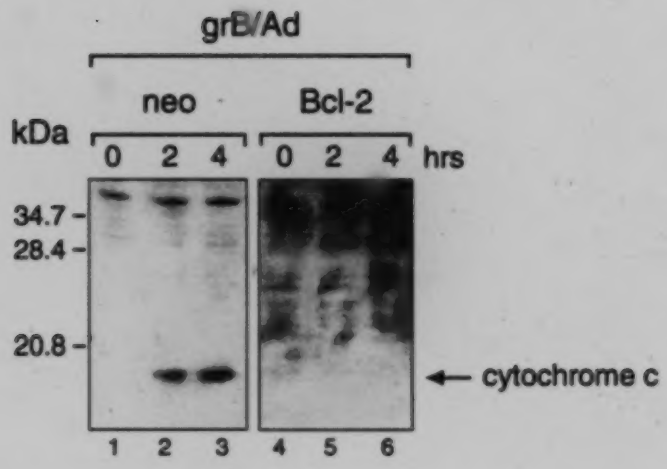


Figure 6-2 Bcl-2 blocks cytochrome c release in response to treatment with granzyme B and adenovirus.

Jurkat targets stably transfected with empty vector (neo) or with Bcl-2 were treated with granzyme B (1 µg/ml) and adenovirus (MOI=10 pfu/cell) at 37°C for the times indicated followed by separation of the membrane fraction (including mitochondria) from the cytosolic fraction. The cytosol was then assayed for the presence of cytochrome c by western blotting using a mouse monoclonal anti-human cytochrome c antibody as indicated in **Chapter 2 (section 2.3.4)**.

in vitro system utilizing purified granzyme B and adenovirus or granzyme B and perforin, all features of apoptosis examined were blocked by Bcl-2.

6.2.2 Bcl-2 Delays but Does Not Block $\Delta\Psi_m$ In Vivo

To examine the effects of Bcl-2 on mitochondria in vivo, GFP-containing targets that over-expressed Bcl-2 were generated (similar to those generated for **Chapter 3**), pre-loaded with the fluorescent mitochondrial dye DASPEI and exposed to hCTL (**Figure 6-3**). The use of GFP-containing targets allowed for easy separation of targets and effectors by flow cytometry. Control GFP-expressing Jurkats (JpEGFP), BMGneo vector only controls (JpEGFPneo) and two JpEGFP clones over-expressing Bcl-2 (JpEGFPBcl-2 clones 2 and 3) were examined. At all effector to target ratios examined (1:1, 0.5:1 and 0.25:1), the JpEGFP and JpEGFPneo targets experienced virtually identical levels and kinetics of DASPEI loss. DASPEI negative cells appeared within 15 minutes following the addition of hCTL and ~60-80% of the target cell population were DASPEI negative by two hours. Thus, consistent with data already presented (**Chapter 5, Figures 5-2 and 5-3**), control cells experienced $\Delta\Psi_m$ loss following treatment with hCTL.

With the JpEGFPBcl-2 clones, DASPEI negative cells appeared within 15 minutes following the addition of hCTL (**Figure 6-3**). However, for a 1:1 effector to target ratio, 20-30% of the JpEGFP and JpEGFPneo control cell populations were DASPEI negative whereas only ~5% of the JpEGFPBcl-2 populations were DASPEI negative. By 30 minutes, ~50% of the control cell populations had lost $\Delta\Psi_m$. This contrasts with the JpEGFPBcl-2 targets in which ~10% of the population had lost $\Delta\Psi_m$ at the same time point. Interestingly, however, by one hour post-treatment, the number of DASPEI negative JpEGFPBcl-2 cells was on the rise and by two hours, the population of Bcl-2 over-expressing cells which had lost $\Delta\Psi_m$ was ~50% versus 60-80%

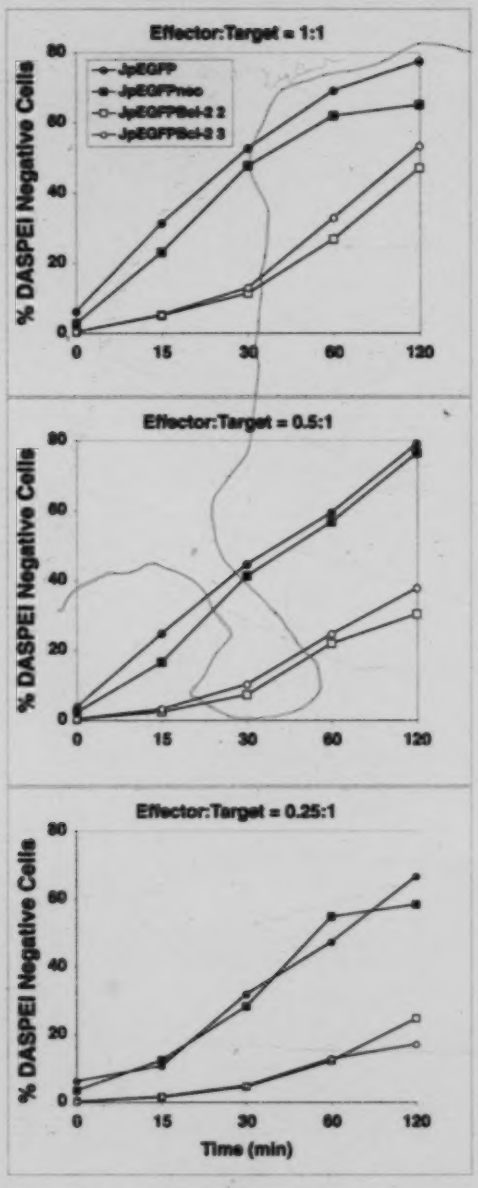


Figure 6-3 Bcl-2 delays $\Delta\Psi_m$ loss in response to treatment with hCTL.

GFP-expressing Jurkat targets (parental JpEGFP, vector-only JpEGFPneo and Bcl-2 over-expressing JpEGFPBcl-2 clones 2 and 3) were pre-loaded with 50 mM DASPEI and then incubated at 37°C with hCTL at the effector to target ratios (hCTL:JpEGFP) indicated. $\Delta\Psi_m$ loss as measured by DASPEI loss was then determined by flow cytometry.

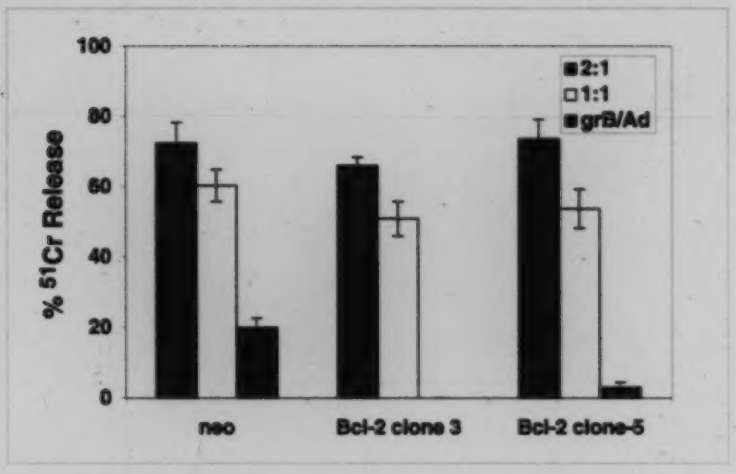
for the control cell populations. As the effector to target ratios decreased (0.5:1 and 0.25:1), the inhibitory effects of Bcl-2 on DASPEI loss became more pronounced and of longer duration. At 0.5:1, ~80% of the control cells had lost $\Delta\Psi_m$ by two hours while Bcl-2 lowered this number to ~35-40%. At 0.25:1, ~60% of control cells were DASPEI negative by two hours whereas Bcl-2 reduced this number to at most 20%.

Thus, in vivo, Bcl-2 primarily caused a delay in the kinetics of $\Delta\Psi_m$ loss rather than preventing $\Delta\Psi_m$ loss as was seen in vitro with granzyme B and adenovirus or granzyme B and perforin. At lower effector to target ratios, however, this kinetic delay was more pronounced and the inhibitory effects of Bcl-2 of longer duration.

6.2.3 Bcl-2 Does Not Prevent Whole CTL-Mediated DNA Fragmentation or Membrane Damage

We have seen that while $\Delta\Psi_m$ loss was delayed, it still occurred in the Bcl-2 transfected targets following exposure to hCTL (**Figure 6-3**). We wished to know if other features of apoptosis were similarly occurring. The Bcl-2 transfected cells and the vector-only (neo) controls were mixed with hCTL at either a 2:1 or a 1:1 effector to target ratio and assessed for membrane damage using a ^{51}Cr release assay or DNA fragmentation using a [^{3}H]thymidine release assay (**Figure 6-4**). At a 2:1 effector to target ratio, membrane damage was seen to be ~75% whereas a 1:1 ratio gave ~60% in the neo controls (**Figure 6-4A**). Similarly, membrane damage was also approximately 70-80% at 2:1 and ~60% at 1:1 with either of the Bcl-2 clones tested (the same clones were used here that were used for the assays of **Figure 6-1**). As an additional control, these clones were also treated with granzyme B and adenovirus and, as expected, membrane damage was observed in the neo controls (~20%) whereas little or none was seen with the Bcl-2 transfected clones (**Figure 6-4A**).

A.



B.

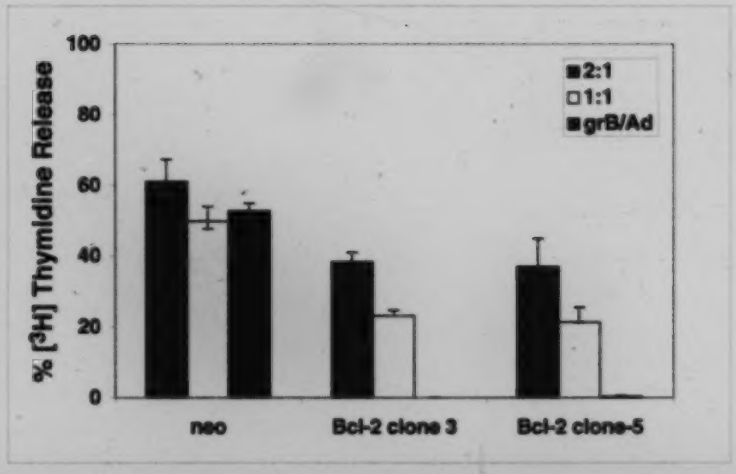


Figure 6-4 Bcl-2 blocks DNA fragmentation and membrane damage in response to granzyme B and adenovirus but not hCTL.

A. Membrane damage is blocked by Bcl-2 in response to granzyme B and adenovirus but not hCTL. Vector-only (neo) or Bcl-2 transfectants were examined for ^{51}Cr release following treatment for four hours with hCTL at either a 2:1 or 1:1 effector:target ratio. Alternatively, cells were treated for the same length of time with granzyme B (1 µg/ml) and adenovirus (MOI=10 pfu/cell). **B.** DNA fragmentation is blocked by Bcl-2 in response to granzyme B and adenovirus but not hCTL. Neo cells and the Bcl-2 transfectants were examined for DNA fragmentation by a [^{3}H]thymidine release assay following treatment with hCTL at either a 2:1 or 1:1 effector to target ratio for three hours at 37°C. Alternatively, cells were treated with granzyme B and adenovirus at the same concentrations as for **A.** Data provided by Michele Barry.

The effects on DNA fragmentation, as measured by [^{3}H]thymidine release, were similar to those seen for membrane damage (**Figure 6-4B**). DNA fragmentation was observed in ~60% of the neo controls at a 2:1 effector to target ratio while at 1:1, ~50% of the population was positive. When exposed to granzyme B and adenovirus, the neo controls were approximately 50% positive for DNA fragmentation. In the presence of Bcl-2 over-expression, however, DNA fragmentation was not observed in either clone in response to granzyme B and adenovirus consistent with the data presented in **Figure 6-1** using TUNEL to measure DNA fragmentation. Interestingly, DNA fragmentation in the Bcl-2 transfectants was not blocked in response to hCTL although the levels of DNA fragmentation were decreased (**Figure 6-4B**). At 2:1, the population of Bcl-2 transfected targets with fragmented DNA dipped to ~40% (a 30% drop) with either clone. At a 1:1 effector to target ratio, DNA fragmentation decreased to ~ 20% (nearly a 60% reduction relative to the neo controls).

Collectively, the data shows that Bcl-2 does not prevent membrane damage in response to hCTL but does retard DNA fragmentation. By contrast, Bcl-2 completely abrogates both membrane damage and DNA fragmentation in response to granzyme B and adenovirus consistent with the flow cytometric data of **section 6.2.1**.

6.2.4 Bcl-2 Retards but Does Not Prevent Granzyme B-Mediated Caspase-3 Cleavage But Does Block Caspase-3 Enzymatic Activity

Bcl-2 appeared to block all apoptotic features of apoptosis examined in our in vitro system with granzyme B and adenovirus/perforin (**Figures 6-1** and **6-2**). At least one published study has shown that Bcl-2 interferes with CTL-mediated caspase-3 activation (Renvoizé et al., 1997). Since caspase-3 is thought to be responsible for at least some of the biochemical and morphological features of apoptosis and since granzyme B can directly activate caspase-3 (Atkinson et al., 1998), we wished to know what effect(s) Bcl-2 had on caspase-3 processing and activation. To investigate this,

vector-only control Jurkats (neo) and Bcl-2-transfected Jurkats (Bcl-2) were treated with granzyme B and adenovirus for up to two hours (**Figure 6-5A**). Caspase-3 cleavage in the neo targets was identical to that observed in parental Jurkats (**Chapter 4, Figure 4-4**). Caspase-3 was processed to p20 and p19 within 30 minutes following the addition of granzyme B and adenovirus (**Figure 6-5A**). Within one hour, p19 was the most prominent cleavage product and by two hours, the p17 product was also seen (**Figure 6-5A**). Similarly, in the Bcl-2 transfectants, pro-caspase-3 was processed to p20 and p19, both of which were also observed within 30 minutes following addition of granzyme B and adenovirus.

In contrast to the neo controls, in the Bcl-2 cells, neither the p20 nor the p19 products increased in abundance throughout the duration of the assay nor was the p17 product observed. Pro-caspase-3 appeared to be cleaved to the active enzyme despite Bcl-2 over-expression. Caspase-3 has been linked with apoptotic morphologies including DNA fragmentation and membrane damage yet Bcl-2 appeared block all measures of apoptosis examined in our in vitro system (**Figures 6-1** and **6-2**). One possibility to explain the apparent lack of caspase-3 function despite zymogen processing would be the absence of caspase-3 enzymatic activity. To examine this, cells were treated as for **Figure 6-5A** and dually processed. Half of the cells were prepared for SDS-PAGE and immunoblotting while the other half was examined for casapse-3-like enzymatic activity using a zDEVD-pNA fluorometric substrate. As seen in **Figure 6-5B**, caspase-3-like enzymatic activity in neo cells increased within 30 minutes following addition of granzyme B and adenovirus and continued to increase throughout the duration of the assay. By contrast, caspase-3-like enzymatic activity was minimal in the Bcl-2 transfectants even out to two hours following treatment. These data indicate that while caspase-3 processing is not mitigated by Bcl-2,

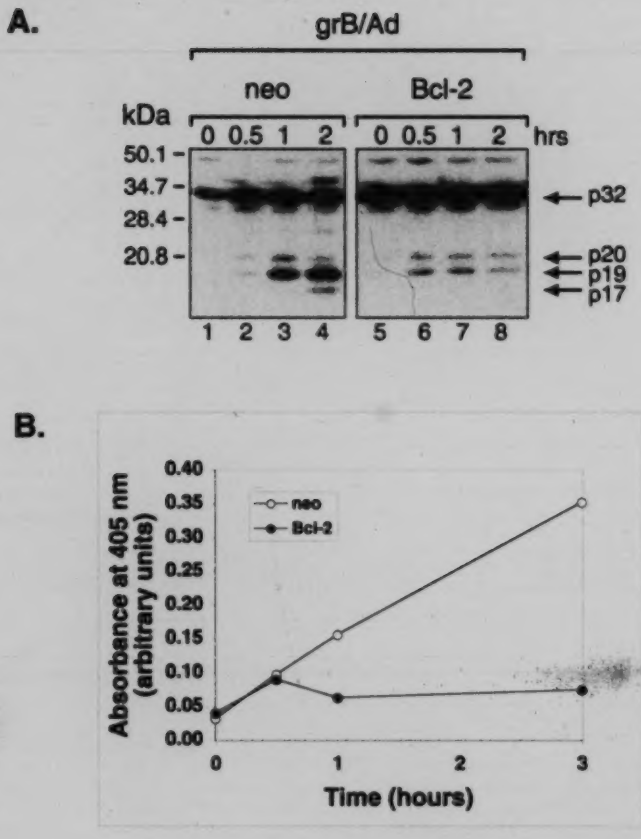


Figure 6-5 Bcl-2 does not prevent caspase-3 processing by granzyme B but does block caspase-3 enzymatic activity.

A. Bcl-2 does not block granzyme B-mediated cleavage of pro-caspase-3. Jurkat targets containing the empty neo vector (neo) or over-expressing Bcl-2 (Bcl-2) were treated with granzyme B and adenovirus at 37°C for the times indicated. Following incubation, cells were separated into membrane and cytosolic fractions as outlined in **section 2.3.4** of **Chapter 2**. The proteins within the cytosolic fractions were then resolved by SDS-PAGE (15% gels) and subsequently transferred to nitrocellulose. Immunoblotting for caspase-3 was performed using affinity purified polyclonal anti-caspase-3 antibodies and ECL. **B.** Bcl-2 inhibits caspase-3 enzymatic activity. Cytosolic fractions from A. were assessed for caspase-3 activity using a zDEVD-pNA substrate. (Data for **B** provided by Ing Swie Goping).

caspase-3-like enzymatic activity is considerably inhibited relative to vector only controls.

6.3 Discussion

The ability of Bcl-2 to regulate CTL-mediated killing remains controversial. Bcl-2 has been shown both to impair and to have no effect whatsoever on killing by CTLs. The data presented here supports the notion that Bcl-2 can block killing in response to granzyme B. All measures of killing assessed, (including DNA fragmentation, phosphatidylserine externalization, gross membrane damage, ROS production, $\Delta\Psi_m$ loss and cytochrome c release), were blocked by Bcl-2 in Jurkat targets in response to granzyme B and adenovirus or granzyme B and perforin (**Figures 6-1, 6-2** and **6-4**). These data support those published by Sutton et al. in mouse myeloid and lymphoid cells which also showed that killing mediated by purified granzyme B and perforin could be blocked by Bcl-2 (Sutton et al., 1997).

In contrast to the inhibition of granzyme B-dependent killing seen in the Bcl-2 transfectants, cell death induced by whole CTL was not blocked by Bcl-2 (**Figures 6-3** and **6-4**). Rather, Bcl-2 caused a decrease in $\Delta\Psi_m$ at early time points that was essentially overcome at later time points. The Bcl-2-induced kinetic delay in $\Delta\Psi_m$ observed in response to hCTL resembled that seen in cells treated with zVAD-fmk prior to the addition of granzyme B and adenovirus or hCTL (**Chapter 4, Figures 4-1A** and **4-2**). Since Bcl-2 appears to be able to block granzyme B-dependent mitochondrial events including $\Delta\Psi_m$ loss, cytochrome c release and killing, the fact that $\Delta\Psi_m$ loss was seen in response to hCTL suggests that granule-derived proteins other than granzyme B are mediating this effect.

Since our hCTL kill predominantly through the granule pathway (**Chapter 3**). Fas-mediated killing is unlikely to be involved. In addition, in Jurkat cells (which are

type II cells; (Scaffidi et al., 1998)), Fas killing predominantly involves the mitochondrial pathway which Bcl-2 can clearly block. Thus, CTL-derived proteins other than granzyme B and FasL must be responsible for these effects. Alternatively, cell to cell killing may be providing a higher concentration of granzyme B on a per cell basis. Higher concentrations of granzyme B may be able to partially compensate for the inhibitory effects of Bcl-2. The fact that at lower effector to target ratios, Bcl-2 provided better protection to targets (Figure 6-3 and 6-4B) supports this possibility.

Bcl-2, by blocking granzyme B-dependent effects, may be able to retard CTL-mediated apoptosis by blocking the activation of caspases downstream of mitochondria. This may impair positive feedback loops directed towards mitochondria and may explain the kinetic delay in $\Delta\Psi_m$ disruption. In addition, the fact that DNA fragmentation was also retarded (but not completely blocked) by Bcl-2 in response to hCTL also supports such a situation (**Figure 6-4B**). Interestingly, for both $\Delta\Psi_m$ loss and DNA fragmentation, the ability of Bcl-2 to block these events was enhanced at lower effector to target ratios (**Figures 6-3** and **6-4B**). We have also obtained similar data for targets transfected with the anti-apoptotic Bcl-2 family member Bcl-X_L in response to granzyme B and adenovirus and hCTL (Barry and Bleackley, unpublished observations). Thus, at low effector to target ratios (as would likely occur in vivo), Bcl-2 and other anti-apoptotic family members may be better able to protect cells from CTL attack. Such a situation may be critically important in tumours where the expression of anti-apoptotic Bcl-2 family members is upregulated.

While $\Delta\Psi_m$ loss and DNA fragmentation was retarded in the Bcl-2 transfectants, little impairment of membrane damage was observed (as measured by ^{51}Cr release; **Figure 6-4A**). Granzyme B in conjunction with adenovirus or perforin can cause membrane alterations that are inhibited by Bcl-2 (**Figure 6-1** and **6-4A**). Adenovirus cannot cause membrane damage alone (**Chapter 3**, **Figure 3-1** and Heibein and

Bleackley, unpublished observations), however, perforin alone can clearly cause cell lysis if provided to targets in sufficient quantities. Such concentrations are achieved at the contact sites between target and CTL (Liu et al., 1995). Therefore, the membrane damage seen in targets despite Bcl-2 over-expression is likely the result of perforin channel-forming activity leading to osmolytic damage. Such a role for perforin despite Bcl-2 over-expression has already been described (Torigoe et al., 1994).

Bcl-2 blocked DNA fragmentation in Jurkats treated with granzyme B and adenovirus or granzyme B and perforin (**Figures 6-1** and **6-4**). A previous study examining the effects of Bcl-2 on granule-mediated killing showed that Bcl-2 prevented cleavage of the classical caspase-3 substrate poly(ADP-ribose) polymerase (PARP) (Renvoizé et al., 1997). This was interpreted to mean that caspase-3 activity was blocked in the Bcl-2 transfectants. These data were consistent with the absence or delay in these cells of classical caspase-3-dependent morphological features of apoptosis such as DNA fragmentation (Wolfe et al., 1999) and membrane damage (Kothakota et al., 1997). We show here that Bcl-2 did not prevent caspase-3 processing as evidenced by the presence of both p20 and p19 fragments (**Figure 6-5A**). However, processed caspase-3 in these cells was not active as determined by its inability to cleave a zDEVD-pNA substrate (**Figure 6-5B**) and the absence of DNA fragmentation in these cells (**Figure 6-4B**). Normally in targets, cleavage to p20 is the direct result of granzyme B enzymatic activity while processing to p19 can be accomplished by caspase-3 itself or another zVAD-fmk-sensitive caspase (Chapter 5 and Atkinson et al., 1998). However, here, despite processing to p20, caspase-3 was not active and so the second cleavage of caspase-3 to give p19 was likely not a result of intrinsic caspase-3 activity. Cleavage of caspase-3 to p19 was not caused by direct granzyme B processing either since cleavage to generate p19 does not occur in the presence of zVAD-fmk in targets treated with granzyme B and adenovirus (**Chapter 5**,

Figure 5-4) or whole CTL (Atkinson et al., 1998). Rather, this processing event is likely to be the result of a caspase or another zVAD-fmk-sensitive protease.

One possible candidate for such a protease is caspase-9. This caspase can be processed by granzyme B in vitro (Duan et al., 1996; Srinivasula et al., 1996) and we have evidence that caspase-9 is also directly cleaved by granzyme B in whole cells (Barry and Bleackley, unpublished observations). However, caspase-9 appears not to be processed in Bcl-2 transfected targets in response to granzyme B and adenovirus. This argues against the involvement of capsase-9 in activating caspase-3 to p19.

An alternative candidate for processing to p19 is caspase-8 considering that this caspase is directly processed by granzyme B in targets (Barry et al., 2000) and since caspase-8 has been shown to process caspase-3 directly (Medema et al., 1997; Scaffidi et al., 1998; Stennicke et al., 1998). We currently do not know the identity of the protein(s) responsible for processing caspase-3 to p19. However, co-transfecting both Bcl-2 and the rabbitpox virus serpin SPI-2 into Jurkat targets could test the involvement of caspase-8. Since SPI-2 is a good inhibitor of caspase-8 (Barry et al., 2000), processing of caspase-3 to p19 in the Bcl-2 over-expressing targets would indicate that caspase-8 is not required for this proteolytic event.

The fact that granzyme B-processed caspase-3 is enzymatically active in an in vitro transcription translation system (Darmon et al., 1995) appears to be at odds with the events observed in vivo in the presence of Bcl-2 in that caspase-3, while processed, was not active (**Figure 6-5**). However, in vivo, processing of pro-caspases, including procaspase-3 and -9 but not pro-caspase-8, and their enzymatic activity are inhibited by the action of a number IAPs (for a recent review on IAPs see Deveraux and Reed, 1999). IAP-mediated inhibition of caspase-3 and -9 is thought to occur through the interactions of IAP baculoviral inhibitory repeat domains (BIRs) binding to caspases, thereby blocking caspase activity.

Recently a mammalian protein called Smac/DIABLO was identified that relieves IAP-mediated caspase repression (Du et al., 2000; Verhagen et al., 2000). Smac/DIABLO is localized to mitochondria but following an apoptotic stimulus is released to the cytosol where it interacts with IAPs (Du et al., 2000; Verhagen et al., 2000). Smac exists as an arc-shaped homodimer and interacts with the BIR domains of IAPs, presumably disrupting their inhibitory interactions with caspases to release the repression of caspase enzymatic activity (Chai et al., 2000). The effects of Bcl-2 expression on caspase-3 processing and activation in our system could be explained by the involvement of IAPs and Smac/DIABLO. The release of Smac/DIABLO is blocked by Bcl-2 (Verhagen et al., 2000) and Bcl-2 blocked granzyme B-mediated cytochrome c release in our system (**Figure 6-2**). Thus while granzyme B may be able to directly process caspase-3, IAP repression could block caspase-3 enzymatic activity. Removal of this repression would require Smac/DIABLO that is not released from mitochondria in the presence of Bcl-2 over-expression. Processing of caspase-3 to p19 could be the result of caspase-8 activity since caspase-8 activity is not inhibited by the known human IAPs (Deveraux et al., 1998; Roy et al., 1997).

The data presented here are consistent with a model in which granzyme B acts on mitochondria either directly or indirectly to cause cytochrome c release, thereby inducing caspase-3 processing following apoptosome formation (**Figure 6-6**). The mitochondrial pathway to caspase-3 activation is antagonized by Bcl-2, which prevents cytochrome c release and apoptosome formation. Nevertheless, granzyme B can also directly processes pro-caspase-3 to generate the p20 fragment which is further processed by a zVAD-fmk-sensitive caspase or other protease. Since Bcl-2 blocks cytochrome c release, caspase-9 is not activated and therefore is unlikely to be responsible for cleavage of caspase-3 to p19. Further, although granzyme B may be able to cleave and activate pro-caspase-9 directly, caspase-9 may not be active as a

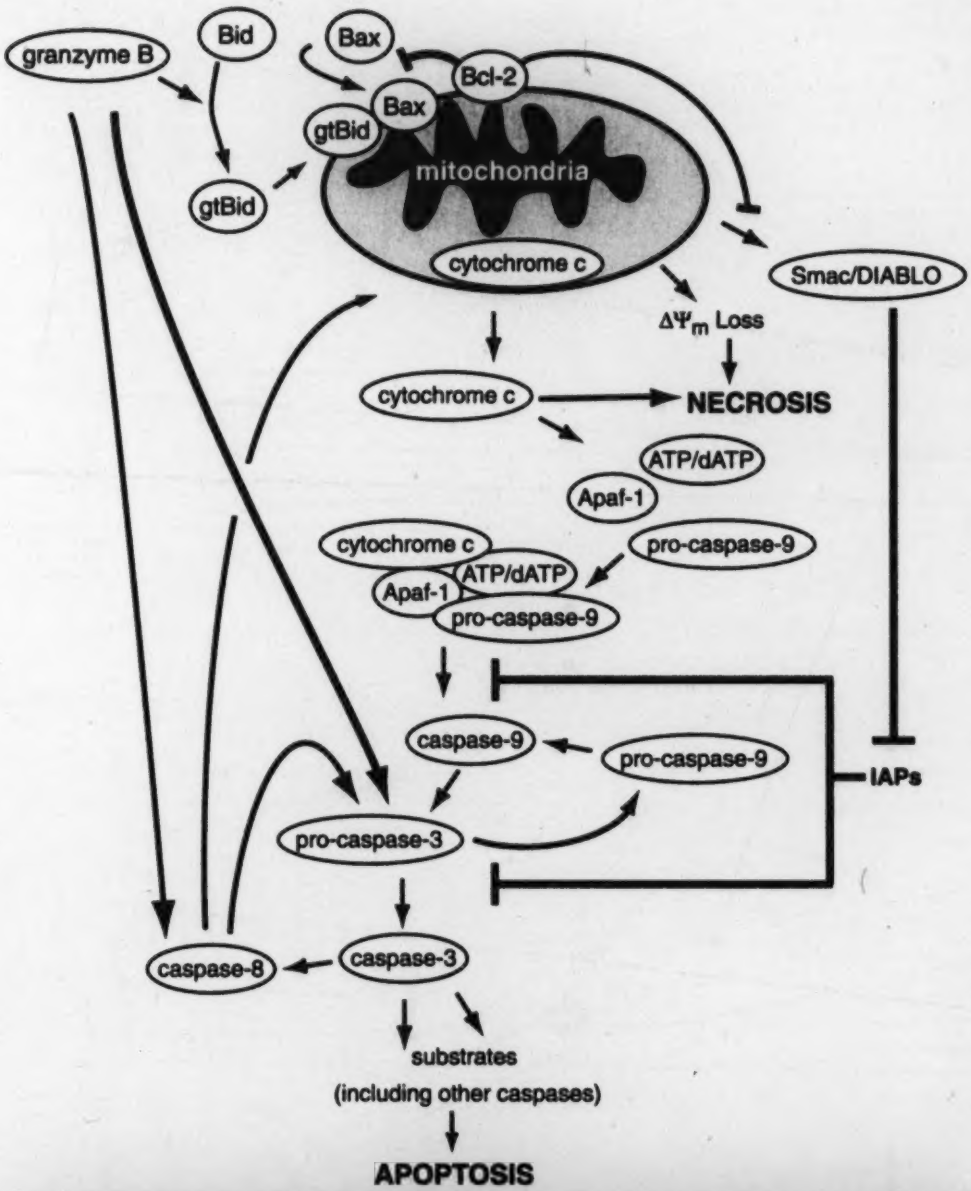


Figure 6-6 Model for regulation of granzyme B-mediated caspase-3 activation by Bcl-2.

Granzyme B activates caspase-3 directly but also activates caspase-3 indirectly through a mitochondrial pathway that is antagonized by Bcl-2. Bcl-2 also blocks the release of Smac/DIABLO which prevents the removal of repression by IAPs of the enzymatic activity of caspase-3 and casapse-9 despite the direct processing of pro-caspase-3 by granzyme B. Granzyme B can also activate caspase-8 which can contribute to caspase-3 processing.

result of IAP-mediated repression. Processed caspase-3 is also not enzymatically active because of the action of IAPs. In addition to cytochrome c, Bcl-2 also blocks the release of a number of apoptogenic proteins including Smac/DIABLO that normally removes IAP repression of caspases. Such a model provides an explanation for the absence of caspase-3 enzymatic activity and the morphological consequences of this activity despite the apparent processing of caspase-3 to an enzymatically active form. It is important to note that such a model, while appropriate for granzyme B-mediated cell death, clearly cannot explain the inability of Bcl-2 to prevent killing mediated by whole CTL.

Although the roles of IAPs and Smac/DIABLO are purely speculative at this juncture, their involvement nevertheless fits well with the available data. In addition, since many reagents are becoming available for these proteins, their involvement in Bcl-2-antagonized granzyme B-mediated apoptosis will be readily testable in the future.

Chapter 7
Summary and Future Directions

Chapter 7: Summary and Future Directions

7.1 Summary of Thesis

7.1.1 Caspase-Independent Granzyme B-Mediated Killing

At the time I began the studies presented in this thesis, the paradigm of granzyme B-dependent killing involved its direct action on caspases, and in particular caspase-3, to effect target cell apoptosis. Indeed, there now is much experimental evidence to support such a direct role for granzyme B in caspase activation. However, reports of granule-mediated killing occurring independently of caspases were beginning to surface (Sarin et al., 1998; Sarin et al., 1997). In addition, mitochondria were coming to the forefront of apoptosis research as organelles with a central coordinating role in the initiation events of apoptosis. Moreover, in some situations, mitochondrial pathways could be stimulated in the absence of caspase activation, although ultimately, triggering these pathways led to caspase activation downstream of mitochondria. We therefore sought a connection between the caspase-independence of granzyme B-mediated killing and mitochondria.

When added to Jurkat targets, granzyme B in conjunction with perforin or a replication deficient adenovirus was shown to cause efflux of cytochrome c to the cytosol as well as loss of $\Delta\Psi_m$ in addition to classical indicators of apoptosis such as DNA fragmentation and membrane damage. DNA fragmentation was clearly caspase-dependent as it was completely blocked by the addition of either the broad-spectrum caspase inhibitor zVAD-fmk or the inhibitor of caspase-3-like caspases, zDEVD-fmk. Similar effects were seen when whole CTLs were added to Jurkat targets. However, the loss of $\Delta\Psi_m$ and cytochrome c release were not dependent upon the activity of caspases since neither zVAD-fmk nor zDEVD-fmk was able to block these events. In

addition, while caspase inhibitors prevented targets of granzyme B-mediated killing from dying with apoptotic morphologies, the targets ultimately died through a more necrosis-like process. Hence we showed that granzyme B could impinge upon mitochondrial pathways to induce cell death even in the absence of caspase activation consequently explaining the caspase-independent killing that had been previously observed.

7.1.2 The Role of Bid and Bax in Granzyme B-Mediated Killing

The studies of **Chapter 3** indicated that granzyme B may have been acting on mitochondria directly, or indirectly through proteolytic action on another intermediate to elicit cytochrome c release. To address this, mitochondria were isolated from Jurkat cells, treated with granzyme B and examined for the loss of cytochrome c. Granzyme B alone was unable to induce the efflux of cytochrome c from isolated mitochondria unless added in conjunction with a cytosolic extract. This suggested that a cytosolic factor or factors were additionally required for granzyme B to elicit this response. The pro-apoptotic Bcl-2 family member Bid has been shown to be responsible for the loss of cytochrome c in the Fas system following its cleavage by caspase-8 and subsequent translocation to mitochondria (Li et al., 1998; Luo et al., 1998). Since it was known that granzyme B could cleave Bid in vitro (Li et al., 1998) and since granzyme B-truncated Bid (gtBid) was found in targets treated with granzyme B (Barry et al., 2000), we investigated Bid as the cytosolic factor responsible for granzyme B-mediated cytochrome c release. We found that recombinant Bid in conjunction with granzyme B, but not Bid alone, was able to induce cytochrome c release from isolated mitochondria. In whole cells, granzyme B induced the cleavage of cytosolic Bid to the 14 kDa gtBid product that was found associated with mitochondria.

To further elucidate the mechanism of granzyme-B-dependent cytochrome c release, we investigated the role of another pro-apoptotic Bcl-2 family member, Bax, in this process. Bax was found to become associated with mitochondria through its integration into the mitochondrial membrane. In response to treatment with granzyme B and adenovirus, Bax was not found integrated into mitochondrial membranes nor was cytochrome c release observed despite the processing and translocation of gtBid to mitochondria. Although the data presented in **Chapter 4** do not conclusively point to an absolute dependence of cytochrome c release on Bax in the granzyme system, increasing experimental evidence in the literature supports a model where Bid recruitment of Bax to the mitochondrial membrane is absolutely required for cytochrome c release. The fact that the absence of cytochrome c release in response to Bcl-2 transfection correlates with the absence of Bax recruitment to the mitochondrial membrane, strongly supports existing evidence that Bid recruits Bax to mitochondria to induce cytochrome c efflux.

7.1.3 Caspases Accelerate CTL-Mediated Killing

While granzyme B is able to induce mitochondrial dysfunction and cell death in the absence of caspase activation (**Chapter 3**), the reality in a physiological setting is that caspases are activated. We examined what role caspase activation may contribute to granzyme B-dependent killing. In the absence of caspase activation, cells do not die with a morphology characteristic of apoptosis. DNA fragmentation is not observed nor are phosphatidylserine externalization or reactive oxygen species production seen (**Chapter 3**). However, it was observed that $\Delta\Psi_m$ loss, although ultimately not blocked by caspase inhibition, followed delayed kinetics suggesting that caspase activation served to accelerate this event. This kinetic delay in $\Delta\Psi_m$ loss was only observed with the broad-spectrum caspase inhibitor, zVAD-fmk. The inhibitor of caspase-3-like caspases, zDEVD-fmk, did not significantly affect the kinetics of $\Delta\Psi_m$ loss. Collectively,

the inhibitor data suggest that initiator rather than effector caspases contribute to this effect.

Caspase-3 processing was also examined in response to granzyme B and adenovirus. Granzyme B was found to directly process targets to p20 consistent with previously published data showing a similar processing of caspase-3 when killing was mediated by whole CTL (Atkinson et al., 1998). Processing of caspase-3 to p19 and p17 was found to be caspase-dependent although the identity of the caspase(s) responsible for this processing remains unknown.

7.1.4 Bcl-2 Retards Caspase-3 Cleavage by Granzyme B and Blocks the Appearance of Caspase-3 Enzymatic Activity

The clear involvement of mitochondria in CTL-mediated killing coupled with the known roles of mitochondrially-localized Bcl-2 in regulating apoptotic events such as cytochrome c release and $\Delta\Psi_m$ loss imply a role for Bcl-2 in granzyme B-mediated target cell death. However, regulation by Bcl-2 continues to be a controversial aspect of granule-mediated killing. A variety of studies suggest that Bcl-2 completely or partially blocks granule-dependent death in some situations while in others, provides no protection whatsoever (Chiu et al., 1995; Renvoizé et al., 1997; Sutton et al., 1997; Torigoe et al., 1994).

We examined what effect Bcl-2 over-expression had on Jurkat targets treated with granzyme B and whole-CTLs. It was found that Bcl-2 blocked all measures of apoptosis in response to treatment with granzyme B and perforin or granzyme B and adenovirus including DNA fragmentation, membrane damage, ROS production, cytochrome c release and $\Delta\Psi_m$ loss. Interestingly, Bcl-2 did not block processing of pro-caspase-3 consistent with direct cleavage by granzyme B. However, Bcl-2 did inhibit the enzymatic activity of caspase-3. A speculative model was proposed to

account for this effect in which the enzymatic activity of caspase-3 is normally repressed by the action of IAP proteins. Release of this activity, despite the direct processing of pro-caspase-3 by granzyme B requires the anti-IAP-repressive effects of Smac/DIABLO that is normally sequestered in mitochondria. However, Bcl-2 prevents the release mitochondrial proteins, including Smac/DIABLO and this prevents the removal of IAP repression. Hence, despite direct processing of pro-caspase-3 by granzyme B, enzymatic activity requires that IAPs be inactivated.

In addition, data presented here showed that while Bcl-2 can block granzyme B-mediated killing, it cannot prevent killing by whole-CTLs, implying that other granule-derived proteins other than granzyme B are responsible for these effects.

7.2 Future Directions

7.2.1 Role of Caspase-8 in Granzyme B-Mediated Killing

Although caspase-8 is not absolutely required for granzyme B-mediated killing (Barry et al., 2000), it is nevertheless processed in targets in response to treatment with granzyme B and adenovirus (Barry et al., 2000) or granzyme B and perforin (Medema et al., 1997). In addition, capsase-8 appears to be a candidate for the caspase responsible for additional processing of caspase-3 to p19. Since we have Jurkat targets expressing the rabbitpox virus serpin, SPI-2, which is an excellent caspase-8 inhibitor, it will be possible to investigate the role of caspase-8 in this process.

7.2.2 Involvement of IAPs and Smac/DIABLO in Granzyme B-Mediated Target Cell Death

In **Chapter 6**, a model was proposed to account for the direct processing of pro-caspase-3 by granzyme B, yet with an absence of caspase-3 enzymatic activity when targets over-expressing Bcl-2 were treated with granzyme B and adenovirus.

This model proposed that caspase-3 enzymatic activity is inhibited by cellular IAPs despite processing of pro-caspase-3 by granzyme B. The removal of IAP repression requires Smac/DIABLO that must be released from mitochondria to access cytosolic IAPs. In the presence of Bcl-2, Smac/DIABLO remains in mitochondria and IAP repression is not removed. Hence pro-caspase-3 is directly processed to an active enzyme by granzyme B but IAPs keep caspase-3 inactive.

The participation of Smac/DIABLO and IAPs in this model is speculative but their involvement is readily testable. Antibodies are available for Smac/DIABLO (Du et al., 2000) and the release of this protein from mitochondria could be assessed using the same assay for cytochrome c release that has already been established (**Chapter 3**). Presumably, Smac/DIABLO will be found to exit mitochondria, as does cytochrome c, in response to granzyme B treatment. Additionally, Bcl-2 should block this event, correlating with the absence of caspase-3 enzymatic activity in these cells.

Reagents for the known human IAPs have been available for some time and so it will also be possible to examine the interactions between caspases, Smac/DIABLO and granzyme B.

7.2.3 Why Does Bcl-2 Not Block Killing by Whole CTL?

Bcl-2 clearly blocked apoptosis mediated by granzyme B and adenovirus or granzyme B and perforin (**Chapter 6**). However, Bcl-2 appeared to delay rather than abrogate apoptosis induced by whole CTLs suggesting that other granule-derived proteins are able to by-pass Bcl-2 regulation. The functions of many of the granule proteins, including most of the granzymes, remain unrevealed and in some cases have not been investigated. However, some information exists that may support possible contributions from other granzymes.

Granzyme A$^{-/-}$ mice appear to have a small but reproducible defect in both DNA fragmentation and membrane damage which can largely be accounted for by the presence of granzyme B and granzyme K (Shresta et al., 1997). However, Granzyme A-induced cytotoxicity has been reported to be both Bcl-2 insensitive and caspase-independent (Beresford et al., 1999). Thus granzyme A could account for at least some of the Bcl-2 insensitive killing observed in our system.

Granzyme K in conjunction with perforin has been reported to induce apoptosis characterized by cytochrome c release and $\Delta\Psi_m$ loss (MacDonald et al., 1999). However, these events appeared to be blocked by Bcl-2 and so may not be directly relevant to CTL-mediated killing.

Granzyme H is a neutral serine protease with chymase activity (Edwards et al., 1999) that, like granzyme B, is inhibited by DCI. The gene for granzyme H is related to cathepsin G and granzyme B and the protein appears to enter cells through receptor-dependent endocytosis similar to granzyme B (Edwards et al., 1999; Haddad et al., 1991). However, substrates of granzyme H are not known and its involvement in apoptosis remain elusive.

Little is known about granzyme C, however, the expression of anti-sense granzyme C oligonucleotides in T cell clones prior to their adoptive transfer into mouse kidney diminished tubular cellular destruction by these cells (Bailey and Kelly, 1997). This suggests that granzyme C contributes to CTL-mediated cytotoxicity at least in some cell types.

Although much is known about granzyme B-mediated killing, little is known about the involvement of other granzymes. However, since Bcl-2's effects in vivo appear to be limited to protection from granzyme B and possibly granzyme K, the roles of the other granzymes requires closer scrutiny.

An alternative explanation for the absence of protection by Bcl-2 from CTL-dependent killing could be based on the concentration of granzyme B. Bcl-2 blocked granzyme B-mediated killing and at lower effector to target ratios appeared to provide increased protection from CTL-mediated killing. This suggests that perhaps at higher effector to target ratios the concentration of granzyme B was higher on a per cell basis than when purified granzyme B was added exogenously. Experiments are currently underway to investigate the ability of Bcl-2 to protect against higher concentrations of granzyme B in our in vitro system.

7.2.4 Differential Target Cell Responses: Do All Cells Respond to Granzyme B-Mediated Killing in the Same Way?

One of the key issues that helped define the involvement of the mitochondrial pathway in Fas-mediated killing was the differential responses of varying targets to Fas receptor ligation (Peter and Krammer, 1998). Following Fas receptor oligomerization, type II cells activate the mitochondrial pathway whereas type I cells do not. Jurkats have been designated as type II since the mitochondrial pathway predominates in these cells in response to Fas receptor ligation and since Bcl-2 can block killing in these targets (Scaffidi et al., 1998). In response to granzyme B-mediated killing, the mitochondrial pathway is also activated in Jurkats and is also inhibited by Bcl-2. However, it is not currently known if all cell types respond to granzyme B-mediated killing as do Jurkats. Different cell-types likely have different expression levels of proteins involved in cell death such as caspases and members of the Bcl-2 family. Since a variety of apoptotic assays are now established in the lab, it will be instructive to completely examine the effects of granzyme B-mediated cell death on a variety of cell types. Such an examination may be extremely relevant for the development of therapeutic approaches in future considering that many viruses have evolved myriad

mechanisms for blocking apoptosis in host cells and since tumours often consist of a variety of transformed cell types.

7.3 Concluding Remarks

Our understanding of apoptosis has advanced considerably since the first morphological descriptions of Kerr, Willie and Currie in the early 1970s. Not bad for a process that was deemed by some to be an artifact of the histochemical procedures used rather than a true physiological phenomenon. Apoptosis is now recognized as an occurrence requiring specialized proteins to carry out specialized functions in a highly regulated fashion. Many of these proteins have been identified and the functions deciphered. However, the roles of many others have not been defined and doubtless numerous additional apoptotic proteins have yet to be unearthed. Nevertheless, it is clear that the granule-dependent pathway of CTL-mediated killing impinges upon pre-existing death pathways within target cells. Data presented in this thesis show that granzyme B can activate caspases either directly, or through mitochondrial pathways. Although many of the mechanistic aspects by which granzyme B influences mitochondrial events remains to be clarified, the fact that granzyme B impinges upon multiple pathways to induce apoptosis must be taken into account when considering future therapeutic interventions. Clearly, there is still a great deal of exciting work to be done in the field of CTL-mediated killing and elucidating the mysteries of this process will enhance our knowledge of immune function in addition to ameliorating current treatments for chronic virus-infections, tumours as well as immunosupressive therapies.

Chapter 8
Bibliography

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